FINAL REPORT

DEVELOPMENT OF ALTERNATIVE PRETREATMENT AND BIOMASS FRACTIONATION PROCESSES: LIME PRETREATMENT

NREL SUBCONTRACTOR REPORT

Part I
Laboratory Results

by

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Subcontract No.: XAW-3-11181-03
Under Prime Contract DE-AC02-83CH10093

July 1996

Preface

Man depends on energy in his daily day life. The depletion of traditional energy sources (e.g., fossil fuels) has made numerous scientists and engineers look for an alternative energy source which is safe, clean, effective, and renewable. Most of the alternative energy sources that have been exploited (solar, nuclear, wind, and hydroelectric) produce only electrical energy. However, in the United States, a quarter of the total energy consumed is liquid fuels. Lignocellulosic biomass — an energy source that is safe, clean, effective, and renewable — can be converted to liquid fuels.

Due to the structural features of lignocellulosic biomass, a pretreatment is required before it can be converted to fuels. An effective pretreatment enhances the enzymatic digestibility such that the product yields increase. An economical pretreatment uses inexpensive materials and equipment such that the operating and capital costs are low enough to make the process feasible. An applicable pretreatment requires only a safe and simple process and an easy-to-build plant. And, an environmental pretreatment utilizes a clean technology that recovers chemicals such that no harmful pollution is released. From our research, we believe that lime pretreatment is an effective, economical, applicable, and environmental pretreatment.

This report describes the methods and the results we obtained in the past year to assess the effectiveness of lime pretreatment. I would like to express my sincere appreciation to the National Renewable Energy Laboratory for their financial support and technical recommendations. I would like to thank Dr. Mark Holtzapple, Dr. Carol Holtzapple, and Dr. Richard Davison for their guidance, assistance, and encouragement. Thanks are due to Dr. Barry Burr for his invaluable contributions to this project. I am also thankful to Daryl Hanson for his help during his undergraduate research course.

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Summary

Lime (calcium hydroxide) was used as the pretreatment agent to enhance the enzymatic digestibility of switchgrass. The effect of lime pretreatment on digestibility at various pretreatment conditions has been studied. The optimal pretreatment conditions were: time = 2 h, temperature = 100°C and 120°C, lime loading = 0.1 g/g dry biomass, water loading = 9 mL/g dry biomass. Studies on the effect of particle size indicate that there was little benefit of grinding below 20-40 mesh; even coarse particles (4-10 mesh) digested well. The effect of cellulase loading for enzymatic hydrolysis has been studied and effective hydrolysis was obtained with 5 FPU/g dry biomass. High sugar yields (79% for glucose and 97% for xylose) were obtained due to the lime pretreatment. Under the optimal pretreatment and hydrolysis conditions, the glucose yield was 3 times that of untreated switchgrass, the xylose yield was 11 times, and the total sugar yield was 4.3 times. Using simultaneous saccharification/fermentation, the best ethanol yield was 70% of the theoretical yield. A material balance study showed that little glucan (ca. 10%) was solublized due to the lime pretreatment whereas about 26% of xylan and 29% of lignin became solublized. The lime could not be recovered by carbonating the wash water because the calcium was complexed with soluble organics. To overcome this limitation, three alternative lime recovery processes are presented.

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Introduction

Lignocellulose is the most abundant biomass in the world. It is considered to be a viable alternative energy source because of its availability and ability to be converted to various organic compounds such as alcohols and organic acids. Due to the structrual characteristics of lignocellulosic, its susceptibility to enzymatic hydrolysis is constrained by several aspects such as the presence of lignin (Schurz, 1977), high crystallinity of cellulose (Walseth, 1952; Norkans, 1950; Fan *et al.*, 1980), the acetylation of hemicellulose (Kong *et al.*, 1992), the moisture content of cellulose fibers (Cowling and Brown, 1969), the degree of cellulose polymerization (Cowling and Brown, 1969; Puri, 1984), and the surface area of lignocellulose (Ghose, 1977; Fan *et al.*, 1982; Puri, 1984). To enhance the susceptibility of enzymatic hydrolysis, pretreatment is an essential step in the biomass conversion process. Pretreatment methods can be classified into four categories: physical, chemical, physicochemical, and biological. Since our research concentrates on using lime (calcium hydroxide) as the pretreatment agent, this section will focus on chemical pretreatments, especially alkali pretreatments.

Many chemical pretreatment methods have been developed by pulping industries to remove lignin, or by animal scientists to enhance biomass digestibility. A broad variety of chemicals have been used as chemical pretreatment agents, such as alkalis, acids (Lee *et al.*, 1978; Israilides *et al.*, 1979; Ostrovski and Aitken, 1987), gases (Sullivan and Hershberger, 1959; Dunlap and Chiang, 1980), oxidizing agents (Han and Callihan, 1974; Toyama and Agawa, 1975; Gould, 1985; Ando *et al.*, 1988; Lundeen, 1991), cellulose solvents (Tsao *et al.*, 1978; Tanaka, 1979), and solvent extraction agents (Kleinert, 1974; Bowers and April, 1977; Wright, 1988; Rughani and McGinnis, 1989). Among these chemicals, alkali is the most popular pretreatment agent, not only because it is more economical and it causes less degradation, but also simpler processes can be applied.

Of the alkalis used as pretreatment agents, sodium hydroxide (Feist *et al.*, 1970; Millit *et al.*, 1970; Moore *et al.*, 1972; Anderson *et al.*, 1973; Mandels *et al.*, 1974; Baker *et al.*, 1975; Turner *et al.*, 1990), ammonia and ammonium sulfite (Millet *et al.*, 1970; Waiss *et al.*, 1972; Millet *et al.*, 1975; Morris *et al.*, 1980; Hultquist *et al.*, 1982; Kellens *et al.*, 1983; Brown *et al.*, 1987; Villareal, 1988) are all able to increase biomass digestibility.

The literature on lime pretreatment processes is much smaller than that for sodium hydroxide and ammonia. Table 1 lists the conditions and the results of studies reported in the literature using lime as a pretreatment agent. Rounds and Klopfenstein (1974) studied the effects of several alkalis on the digestibility of corn cobs, including sodium hydroxide, potassium hydroxide, ammonium hydroxide, and lime. They obtained higher *in vitro* digestibility when lime and sodium hydroxide together were used for pretreatment, whereas lime alone did not give any increase in the digestibility. Using the same biomass, Waller and Klopfenstein (1975) obtained higher daily gain and the lowest feed/gain ratio when the biomass was pretreated with 3% sodium hydroxide and 1% lime together. Gharib *et al.* pretreated poplar bark with calcium oxide and reported a 38% to 52% increase in the *in vitro* true digestibility for 150-d pretreatment. Playne (1984) studied the effects of sodium hydroxide, ammonia, and lime as the pretreatment agents for bagasse. High organic matter (OM) yields were obtained for sodium hydroxide and lime, whereas lower yields resulted from ammonia. Felix *et al.* (1990) studied the effects of ensiling and treating soybean straw with sodium hydroxide, ammonia, and lime on ruminant digestibility. Improved digestibility was obtained for ensiled soybean straw whereas dry and unensiled straw did not give any improvement. Nagwani (1992) studied the effects of lime pretreatment on bagasse, wheat straw, and softwood newspaper. The pretreatment conditions were optimized and it was concluded that longer pretreatment times (24 h) were better for lower temperature (50 or 65°C) and shorter pretreatment times (1 h) were better for high temperatures (135°C). The best range of lime loadings were 0.1 and 0.15 g/g dry biomass. The effect of water loading was not significant.

In the previous studies, no significant difference was found in the digestibility between lime and other alkalis as the pretreatment agents. However, from an economical viewpoint, lime is much less expensive than other alkalis. Furthermore, compared to sodium residues, calcium residues have no or little problem for animal feed (Owen *et al.*, 1984). Also, when using an artificial rumen for fermentation, lime will serve as both the pretreatment agent and the neutralizing agent for organic acids. These have made the pretreatment more economical.

Table 1. Lime Pretreatment Conditions Used by Previous Workers

Biomass	Temp. (°C)	Time	Lime Loading (g/g biomass)	Water Loading (g/g solution)	Partical Size	Effect on Digestibility	Reference
Corn Cobs	----	----	0.04	----	----	no effect	(a)
Poplar Bark	ambient	1 or 150 d	0.04 to 0.16	0.6	9.5 mm	increased from 30% to 52%	(b)
Corn Cobs	ambient	14 d	0.04	0.6	ground	improved digestibility	(c)
Bagasse	20	8 d	0.12 to 0.3	0.87	2.25 mm	increased from 19% to 72%	(d)
Soybean Straw	ambient/ frozen	30 d	0.02 to 0.05	0.65	chopped	no effect	(e)
Bagasse	50 to 145	1 to 36 h	0.02 to 0.3	0.86 to 0.95	40 mesh	increased from 6% to 97%	(f)
Wheat Straw	65 to 125	1 to 24 h	0.05 to 0.2	0.86 to 0.95	40 mesh	increased from 9% to 85%	(f)
Softwood Newspaper	60 to 150	1 to 24 h	0.05 to 0.3	0.86 to 0.94	40 mesh	increased from 40% to 57%	(f)

References: (a) Rounds *et al.*, 1975 (b) Gharib *et al.*, 1975 (c) Waller *et al.*, 1975 (d) Playne, 1984 (e) Felix *et al.*, 1990 (f) Nagwani, 1992

Overview of Research Plan

In our past studies, the process of lime pretreatment had been developed and the pretreatment conditions for bagasse had been optimized (Nagwani, 1992). The purpose of this research was to discover more profoundly the effects of lime pretreatment on the enzymatic digestibility for a typical herbaceous biomass, switchgrass, based on our previous results. The specific objectives were:

1. Determine best method of neutralizing lime after pretreatment, i.e., study the inhibitory effects of resulting salts when the lime is neutralized by acetic acid or sulfuric acid. (Note: acetic or sulfuric acid would only be used in a laboratory setting. Industrially, carbon dioxide would be used.)
2. Optimize the pretreatment conditions (i.e., time, temperature, lime loading, water loading, and particle size).
3. Determine the effect of cellulase loading on enzymatic hydrolysis.
4. Perform material balances to determine how much biomass is solubilized due to the lime pretreatment.
5. Use carbon dioxide to recover lime as would be used in industrial processes.
6. Produce ethanol from pretreated biomass using simultaneous saccharification/fermentation.
7. Perform preliminary economic analysis on the lime pretreatment process.

QA/QC

The QA/QC analysis was performed on the aspenwood standard provided by NREL. The NREL *Chemical Analysis & Testing Standard Procedures* No. 001-005 were exactly followed except that 20 μL of sample injecting volume for the HPLC analysis was used, as required by the instructions of Biorad. The analysis was performed at least in duplicate and the arithmetic average of all runs was taken as the recommended value. Table 2 summarizes the results.

Based on our GC measurement, the concentration of the NREL ethanol standard was 30.97 g/L. Based upon HPLC, the glucose standard was 1.03 g/L and based on YSI glucose analyzer, it was 1.01 g/L.

Table 2. QA/QC Analysis of NREL Aspenwood Standard

Components	Run 1	Run 2	Run 3	Recommended Value
Moisture[a]	5.92%	5.85%	5.78%	5.85%
Ash[b]	1.22%	1.18%	1.21%	1.20%
Klason Lignin[b]	25.54%	25.70%	-----	25.62%
Acid-Soluble Lignin[b]	1.92%	2.05%	-----	1.99%
Glucan[b]	42.57%	44.41%	-----	43.49%
Xylan[b]	13.73%	14.74%	-----	14.24%
a. as received basis				
b. 105°C-dried basis				

Acid Study

Purpose

After the biomass is treated with lime, the pH is as high as 11.0, which is incompatible with cellulase; therefore, the lime has to be neutralized. In our previous study, acetic acid was used to neutralize the lime; however, the calcium acetate product inhibited cellulase (Nagwani, 1992). Since calcium sulfate is insoluble and hence may not inhibit cellulase, sulfuric acid was proposed as an alternative to neutralize the lime. The purpose of this study is to determine which neutralization method causes the least inhibition. (Note: the use of acetic or sulfuric acid to adjust the pH is for laboratory convenience. Industrially, we propose to use carbon dioxide.)

Materials and Methods

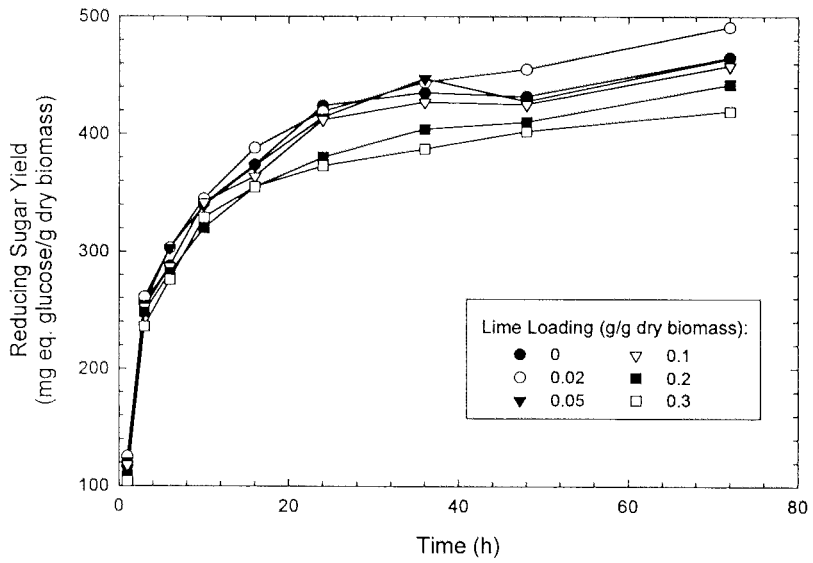
A large sample (ca. 200 g) of switchgrass (-40 mesh) was treated with lime. The pretreatment was performed using the optimal conditions for bagasse (Nagwani, 1992) since the pretreatment conditions for switchgrass had not yet been optimized. The pretreatment conditions were: temperature = 100°C, time = 1 h, lime loading = 0.1 g/g dry biomass, and water loading = 10 mL/g dry biomass. The step-by-step pretreatment procedure is shown in Appendix A, "Lime Pretreatment Procedure for Acid Study." The pretreated switchgrass was washed with fresh distilled water ten times to remove the lime, as explained in Appendix D, "Washing Procedure for Acid Study." The pretreated and washed switchgrass were air-dried and divided into 12 flasks which contained citrate buffer and various lime additions (each lime addition was duplicated). The lime additions explored were: 0, 0.02, 0.05, 0.1, 0.2, and 0.3 g/g dry biomass. The lime added to each flask was neutralized by adding various amounts of acetic acid such that the pH of each flask was 4.8. Then enzymatic hydrolysis was performed at 50°C for 3 days, as described in Appendix C, "Enzymatic Hydrolysis Procedure for Acid Study." The reducing sugar yields at each lime loading were measured as a function of time using the DNS assay (see Appendix E). The reducing sugar yields of the time-zero samples determined the sugar contents of the enzymes and were subtracted from the sugar yields at other time points. The entire procedure was repeated with sulfuric acid as the neutralizing agent.

Results and Discussions

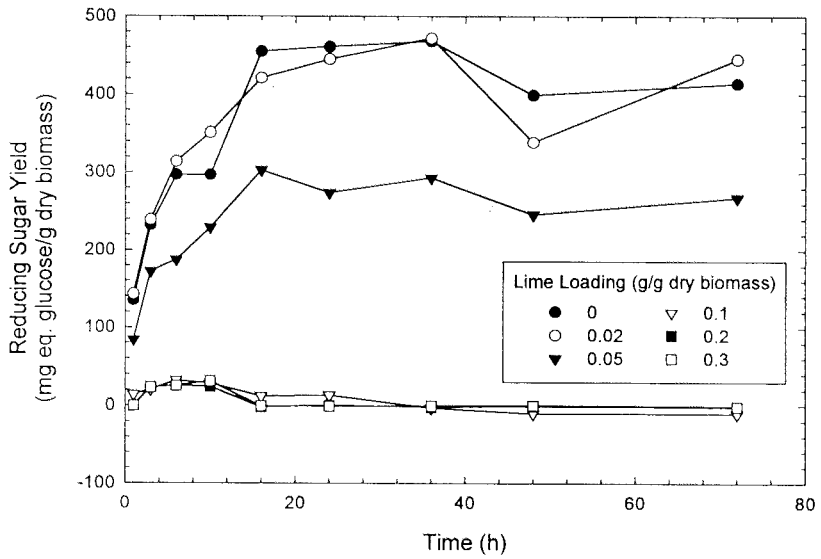
The reducing sugar yields were plotted as a function of time at different lime additions when the lime was neutralized by acetic acid and sulfuric acid, as shown in Fig. 1. The reducing sugar yields decreased as the lime loadings increased during the hydrolysis period. Fig. 2 summarizes the 3-day reducing sugar yields. As anticipated, calcium acetate had an inhibition effect on the enzyme which caused about 10% loss of sugar yields at high lime loadings. However, when the lime was neutralized by sulfuric acid, the 3-day reducing sugar yields were extremely low. This resulted because the calcium sulfate product blocked sulfuric acid from reaching lime at the interior of the particle; that is, the lime was coated by a layer of calcium sulfate. Over a brief period of time, the lime appeared to be neutralized; however, after a long contact, the lime slowly leaked out and raised the pH such that the enzyme activity was destroyed. We observed that the pH rose as high as 9 after three days of hydrolysis.

Conclusions

It is concluded that acetic acid is the appropriate neutralizing agent for lime pretreatment, although the calcium acetate product causes a slight enzyme inhibition.



(a)



(b)

Figure 1. Hydrolysis profiles where lime was neutratlized by different acids. (a) Neutralized by acetic acid (b) Neutralized by sulfuric acid
(Pretreatment conditions: 100°C, 1 h, 0.1 g lime/g dry biomass, 10 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

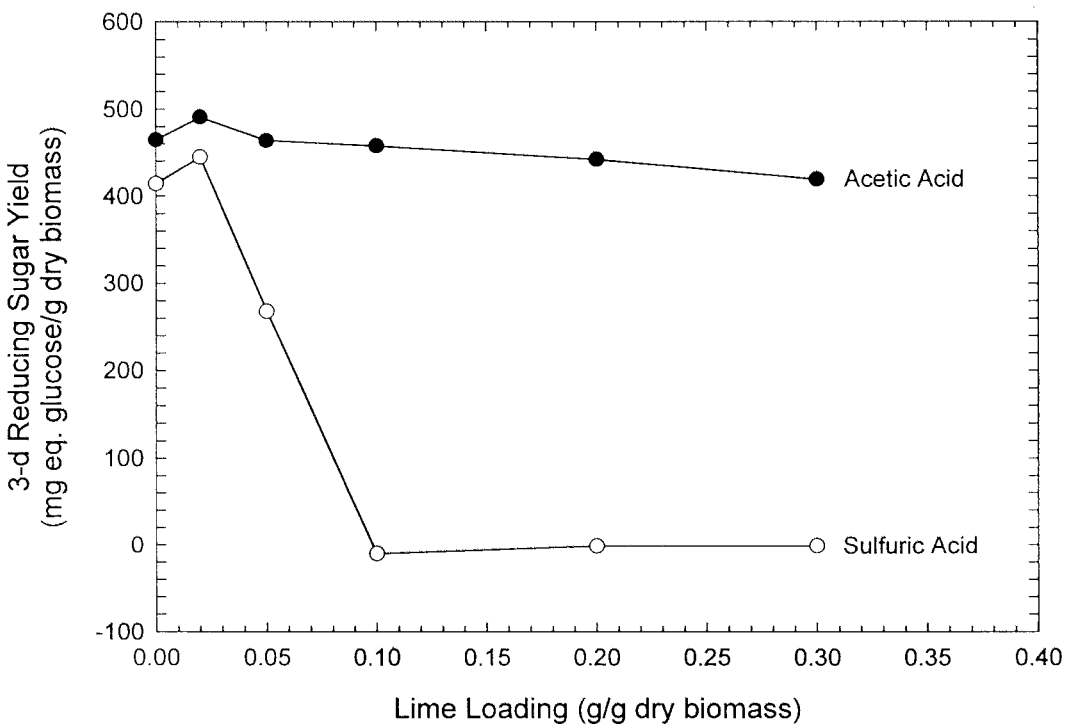


Figure 2. 3-d reducing sugar yields where lime was neutralized by different acids.
(Pretreatment and hydrolysis conditions were the same as in Fig. 1)

Optimization of Pretreatment Conditions

Purpose

A broad survey of reaction conditions were explored to determine which lime pretreatment conditions cause the greatest increase in switchgrass digestibility. A precision estimate of the pretreatment and hydrolysis procedures was performed to check the reproducibility.

Materials and Methods

The pretreatment conditions were systematically varied to explore the effects of process variables (i.e., time, temperature, lime loading, water loading, and biomass particle size) on digestibility. Based on our previous study (Nagwani, 1992), time and temperature had the largest impact on biomass digestibility. Lime loading generally had a critical value (ca. 0.1 g/g dry biomass) below which the digestibility greatly declined and above which the digestibility only increased slightly. Water loading had little effect on the digestibility. Therefore, this study was conducted to hold the low-impact variables (e.g., lime loading, water loading, and particle size) constant while systematically varying the high-impact variables (e.g., time and temperature). Table 3 shows the range of conditions explored.

The initial lime and water loadings (i.e., 0.1 g/g dry biomass and 10 mL/g dry biomass, respectively) were based on our past study (Nagwani, 1992). After pretreatment, the biomass was removed to an Erlenmeyer flask and acetic acid was added to neutralize the lime until the pH reached 4.8. Then an enzymatic hydrolysis was performed at 50°C, where 5 FPU cellulase/g dry biomass and 28.4 CBU cellobiase/g dry biomass were added. After 3 days of hydrolysis, the reducing sugar yield of the biomass was measured using the DNS assay. The sugar content in the enzymes (ca. 4.2 mg eq. glucose/g dry biomass; see "Enzyme Loading Studies") was subtracted from the original sugar yields to determine the actual amounts of sugar produced from the pretreated biomass. After subtracting the enzyme sugars, the yields were multiplied by 1.015 (for the case of a lime loading of 0.1 g $Ca(OH)_2$/g dry biomass) to correct for calcium acetate inhibition and were called "corrected" reducing sugar yields. The correction factor of 1.015 was determined from the results of the "Acid Study." The procedures for lime pretreatment, enzymatic hydrolysis, and DNS assay are shown in Appendices B, C, and E, respectively.

Table 3. The Lime Pretreatment Conditions Explored for Switchgrass

	Time (h)	Temperature (°C)	Lime Loading (g/g dry biomass)	Water Loading (mL/g dry biomass)	Particle Size (Mesh)
Study 1	1 to 24	60 to 130	0.1	10	40 and finer
Study 2	3	100 and 120	0.01 to 0.30	10	40 and finer
Study 3	3	100 and 120	0.1	5 to 15	40 and finer
Study 4	3	100	0.1	9	5 to 80 and finer
Study 5	1 to 3	100	0.1	9	40 and finer

Results and Discussions

Optimization of Pretreatment Time

Figure 3a shows the reducing sugar yields as a function of pretreatment time (i.e., 1, 3, 6, 10, 16 and 24 h) at various temperatures. One hour was obviously insufficient to achieve good sugar yields whereas pretreatment times longer than 3 h had little additional benefit. Therefore, 3 h was temporarily selected as a standard pretreatment time with which other optimization studies were conducted.

A higher resolution study of pretreatment time was performed to focus in the range of 1 to 3 hours. The reducing sugar yields were measured for biomass samples which had been pretreated for 1, 1.5, 2, and 3 hours. Fig. 3b shows that full pretreatment is likely to occur after 2 hours. Therefore, 2 h was selected as the standard pretreatment time.

At short pretreatment times, the method by which the reactors and contents are brought to the pretreatment temperatures may affect the sugar yields. The effects of two methods were explored. In the "heat-up" method, the reactors and contents at room temperature were placed in the oven which was preheated to 20°C above the desired temperature. Time zero was actually 15 minutes after entering the oven to allow for the contents to reach the desired temperature. In the "boil-up" method, the oven was preheated to the desired temperature and the reactors and contents were immersed in boiling water for 5 to 10 minutes before they were placed into the oven. The preheated reactors were placed in the preheated oven at time zero. As shown in Fig. 3b, there is no significant difference between 3-h pretreatments using the "heat-up" and "boil-up" methods.

Optimization of Pretreatment Temperature

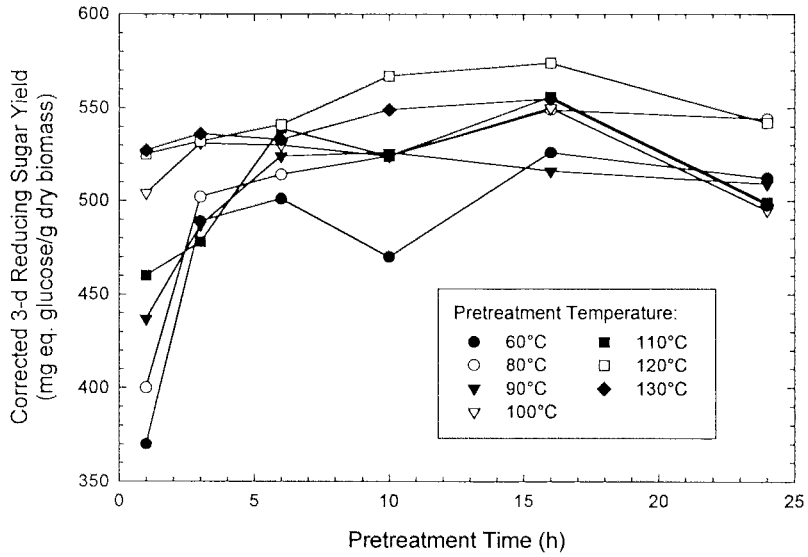
The data in Fig. 3a were replotted in Fig. 4 where the reducing sugar yields were plotted as a function of pretreatment temperature for various pretreatment times. The optimal pretreatment temperature is likely to lie between 100 and 120°C. The best pretreatment resulted after 16 h, but this is excessively long from an economic viewpoint.

Optimization of Lime Loading

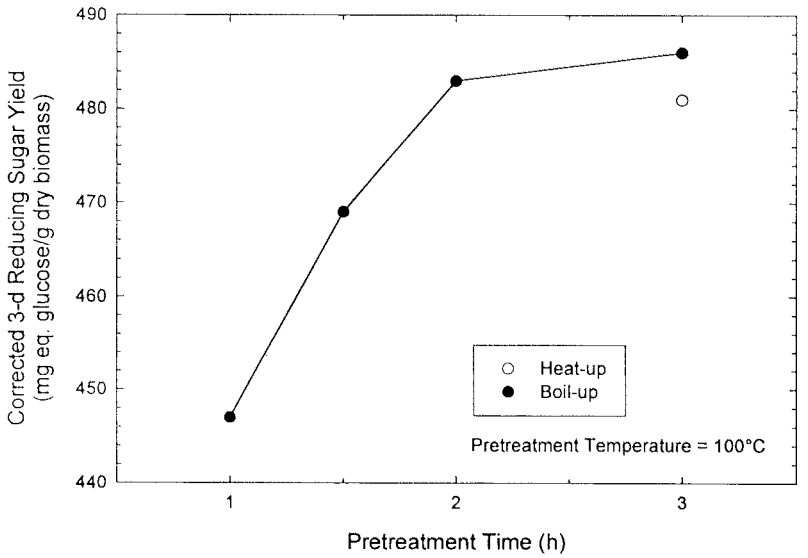
Fig. 5 shows the effect of lime loading at two pretreatment temperatures (100°C and 120°C). The most effective lime loading was 0.1 g/g dry biomass. Although slightly greater sugar yields were obtained at a lime loading of 0.15 g/g dry biomass, the 50% increase in lime consumption cannot be justified. The results at 100°C and 120°C were very similar.

Optimization of Water Loading

Fig. 6 shows the effect of water loading at two pretreatment temperatures (100°C and 120°C). Although water loadings as low as 7 mL/g dry biomass are effective, there is little economic incentive to reduce the water loadings to a bare minimum. Therefore, a water loading of 9 mL/g dry biomass, which has a slight extra benefit (ca. 5% increase in sugar yields), can be used.



(a)



(b)

Figure 3. Optimization of pretreatment time: (a) 1 to 24 h (b) 1 to 3 h. (Pretreatment conditions: 0.1 g lime/g dry biomass, 10 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

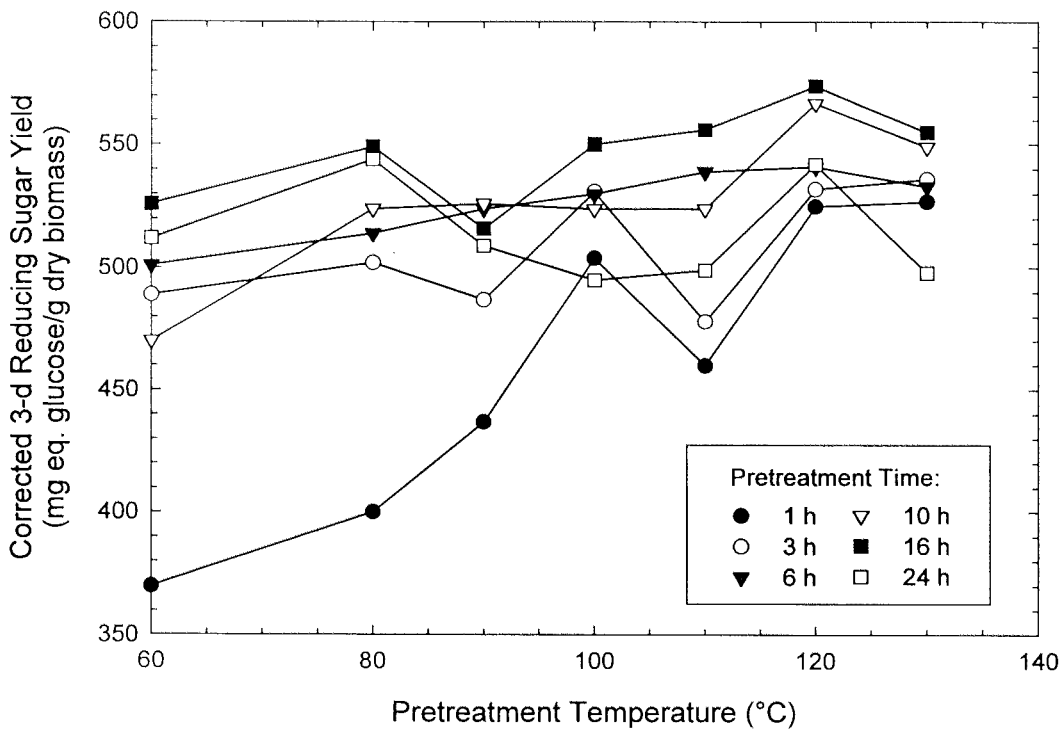


Figure 4. Optimization of pretreatment temperature
(Pretreatment and hydrolysis conditions were the same as in Fig. 3)

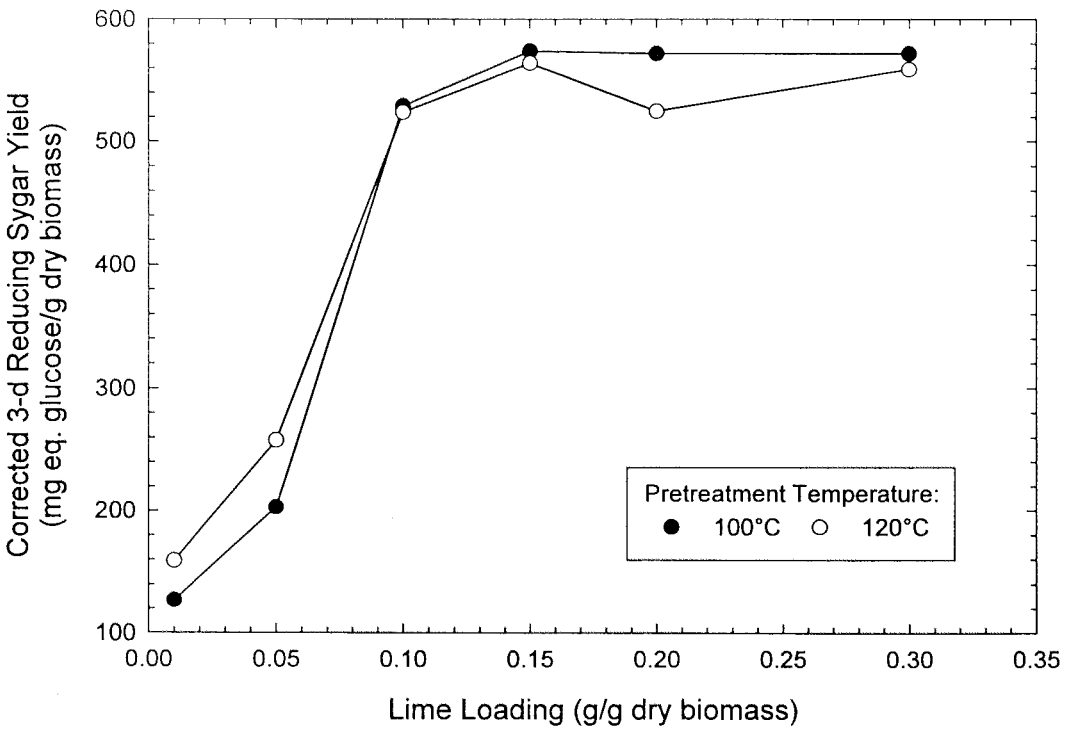


Figure 5. Optimization of lime loading (Pretreatment conditions: 3 h, 10 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

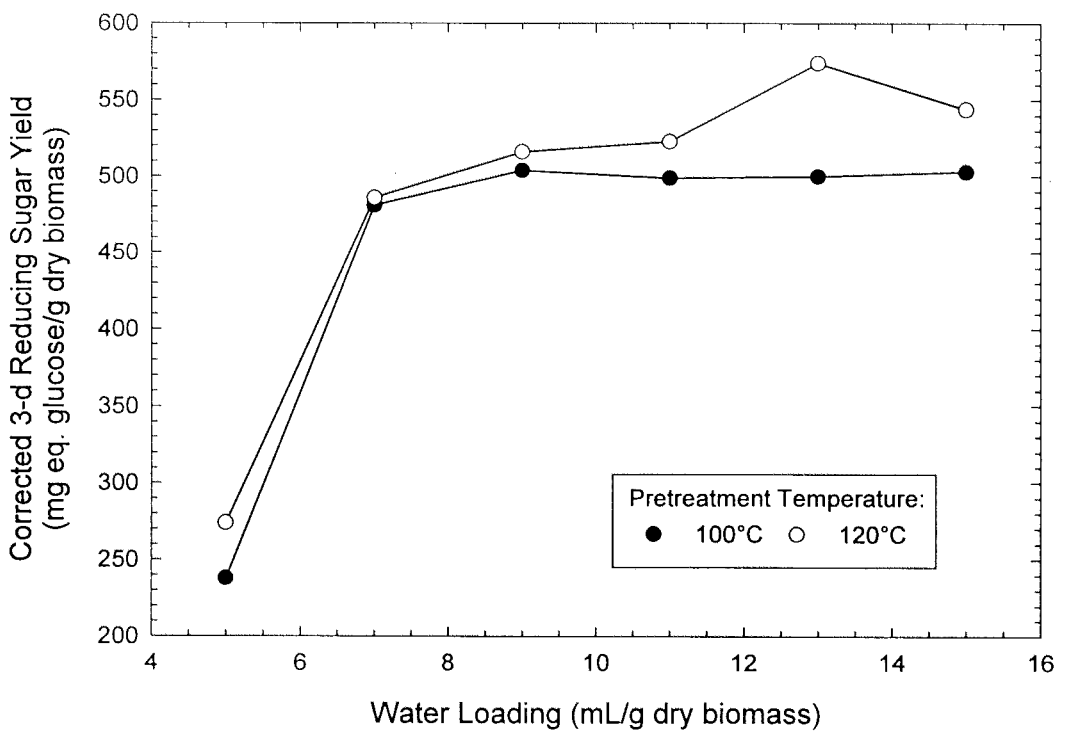


Figure 6. Optimization of water loading (Pretreatment conditions: 3 h, 0.1 g lime/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

Optimization of Particle Size

Fig. 7 shows the effect of biomass particle size on the digestibility. Five different particle sizes were studied. Grinding to less than 20 mesh is sufficient for optimal pretreatment. Even though it was not necessary to grind biomass below 40 mesh, subsequent experiments were performed using particle size less than 40 mesh because there was a large quantity of this material available. Also, it is of a more uniform particle size which reduces variability between experiments.

Method Reproducibility

Internal and external precision tests of the lime pretreatment and enzymatic hydrolysis procedures were performed at 100°C and 120°C. Internal precision was determined by preparing six reactors and pretreating simultaneously and then hydrolyzing simultaneously. External precision tests were accomplished by pretreating the reactors separately and then hydrolyzing simultaneously. Table 4 shows the sample sizes, mean sugar yields, standard deviations, coefficients of variation. Coefficients of variation were calculated using the following formula:

$$\text{Coefficient of Variation} = \frac{\text{Standard Deviation}}{\text{Mean}} \quad (1)$$

According to Table 4, sugar yields are about 7% higher for 120°C pretreatment than for 100°C.

Table 4. Method Reproducibility of Lime Pretreatment and Enzymatic Hydrolysis

Temperature (°C)	Precision Mode	Sample Size	Mean[a]	Standard Deviation[a]	Coefficients of Variation
100	Internal	6	515.3	10.2	0.021
100	External	5	507.9	19.9	0.039
120	Internal	6	558.4	5.1	0.009
120	External	6	543.2	8.6	0.016
a. The unit is mg eq. glucose/g dry biomass.					

Conclusions

Table 5 summarizes the recommended pretreatment conditions for switchgrass.

Table 5. The Optimal Conditions of Lime Pretreatment for Switchgrass

Variables	Recommended Values
Time	2 h
Temperature	100 to 120°C
Lime Loading	0.1 g/g dry biomass
Water Loading	9 mL/g dry biomass
Particle Size	20 mesh and finer

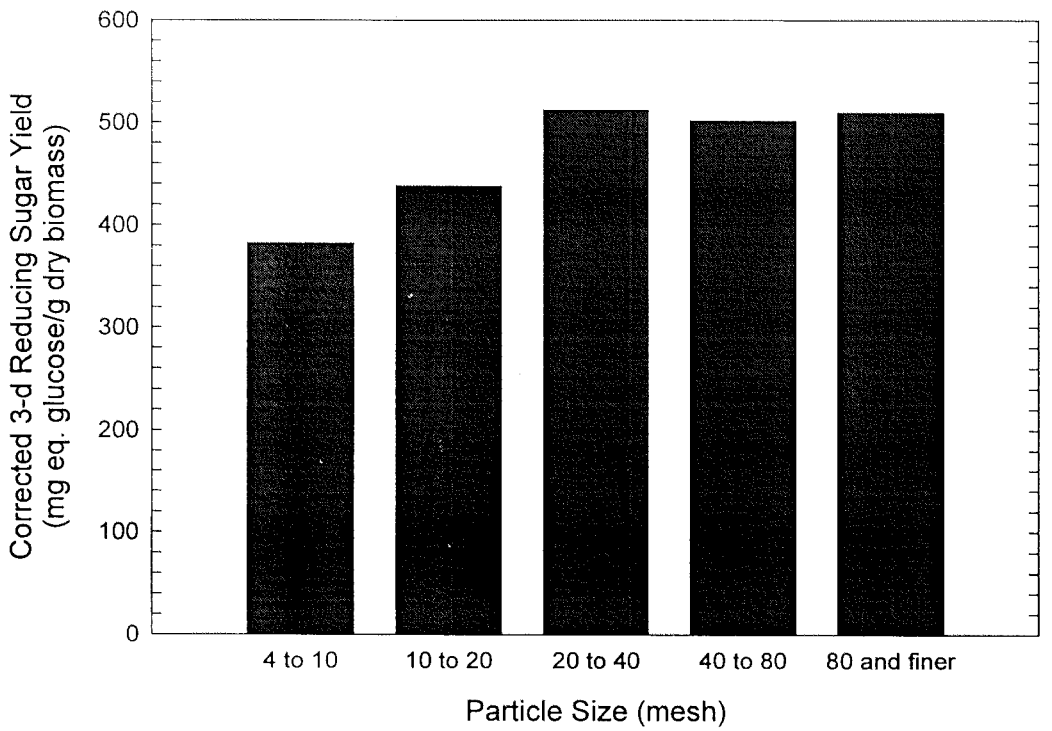


Figure 7. Particle size study (Pretreatment conditions: 100°C, 3 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

Material Balances

Purpose

Material balances were performed to determine how much biomass is solubilized by the lime pretreatment.

Materials and Methods

To remove any solubles, untreated and optimally pretreated switchgrass were repeatedly washed with fresh distilled water until the decanted water became colorless (see Appendix D for the detailed washing procedure). The total weight (105°C dry weight) of the sample was measured before and after pretreatment and wash. The compositions (i.e., glucan, xylan, lignin, and ash) of raw, washed only, and pretreated & washed switchgrass were determined according to the NREL standard procedures No. 002 to 005. The crude protein content of the samples was determined by the Forage Testing Laboratory of Texas A&M University using a modified micro-Kjeldahl procedure which used sulfuric acid-hydrogen peroxide with lithium sulfate and selenium catalysts. Total nitrogen as ammonium was determined colormetrically using a Technicon Autoanalyzer II continuous segmented flow system. The nitrogen value was then multiplied by 6.25 to estimate crude protein.

Results and Discussions

Tables 6 and 7 show the compositions of raw, washed only, and pretreated & washed switchgrass. Table 8 summarizes the loss percentages of each component before and after pretreatment. All the components become more water-soluble due to the lime pretreatment, except ash. Fairly large quantities of lignin, xylan, crude protein, and other components (e.g., extractives) are removed by the lime pretreatment, whereas little glucan is removed; hence, glucan is more stable than other components. About 30% of lignin removed by the lime pretreatment may contribute to the increase of the biomass digestibility. As shown in Fig. 8, the compositions of raw, washed only, and pretreated & washed switchgrass are essentially similar, although the pretreated & washed biomass is slightly enriched in cellulose.

Table 6. Material Balance between Raw and Washed Only Switchgrass

Components	Raw Sample		Washed Sample		Loss	
	Weight[a] (g)	Weight Percentage	Weight[a] (g)	Weight Percentage	Weight[a] (g)	Weight Percentage[b]
Total	20.0348	100.00%	17.4385	100.00%	2.5963	12.96%
Ash	1.1099	5.54%	0.6592	3.78%	0.4507	40.61%
Lignin	4.3476	21.70%	3.8522	22.09%	0.4954	11.39%
Xylan	4.4898	22.41%	4.1080	23.56%	0.3818	8.50%
Glucan	7.5852	37.86%	7.0051	40.17%	0.5801	7.65%
Crude Protein	1.6489	8.23%	1.1248	6.45%	0.5241	31.78%
Others	0.8535	4.26%	0.6892	3.95%	0.1642	19.24%
a. 105°C dry weight.						
b. Based on the initial weight of each component before washing.						

Table 7. Material Balance between Raw and Pretreated[a] & Washed Switchgrass

Components	Raw Sample		Pretreated & Washed Sample		Loss	
	Weight[b] (g)	Weight Percentage	Weight[b] (g)	Weight Percentage	Weight[b] (g)	Weight Percentage[c]
Total	20.0986	100.00%	13.5888	100.00%	6.5098	32.39%
Ash	1.1135	5.54%	0.8058	5.93%	0.3077	27.63%
Lignin	4.3614	21.70%	2.5805	18.99%	1.7809	40.83%
Xylan	4.5041	22.41%	2.9284	21.55%	1.5757	34.98%
Glucan	7.6093	37.86%	6.2930	46.31%	1.3164	17.30%
Crude Protein	1.6541	8.23%	0.6672	4.91%	0.9869	59.66%
Others	0.8562	4.26%	0.3139	2.31%	0.5423	63.34%

a. Pretreatment conditions: Temperature = 120°C, Pretreatment time = 2 h, Lime loading = 0.1 g/g dry biomass, Water loading = 9 g/g dry biomass, and Particle size ≤ 40 mesh

b. 105°C dry weight.

c. Based on the initial weight of each component before pretreatment and washing.

Table 8. Summary of Water-Solubility of Switchgrass Components Before and After Lime Pretreatment

Components	Loss Weight Percentage[a]		Amount Removed by Lime Pretreatment
	Washed Only	Pretreated & Washed	
Total	12.96%	32.39%	19.43%
Ash	40.61%	27.63%	-12.98%
Lignin	11.39%	40.83%	29.44%
Xylan	8.50%	34.98%	26.48%
Glucan	7.65%	17.30%	9.65%
Crude Protein	31.78%	59.66%	27.88%
Others	19.24%	63.34%	44.10%

a. Weight percentage based on the initial weight of each component.

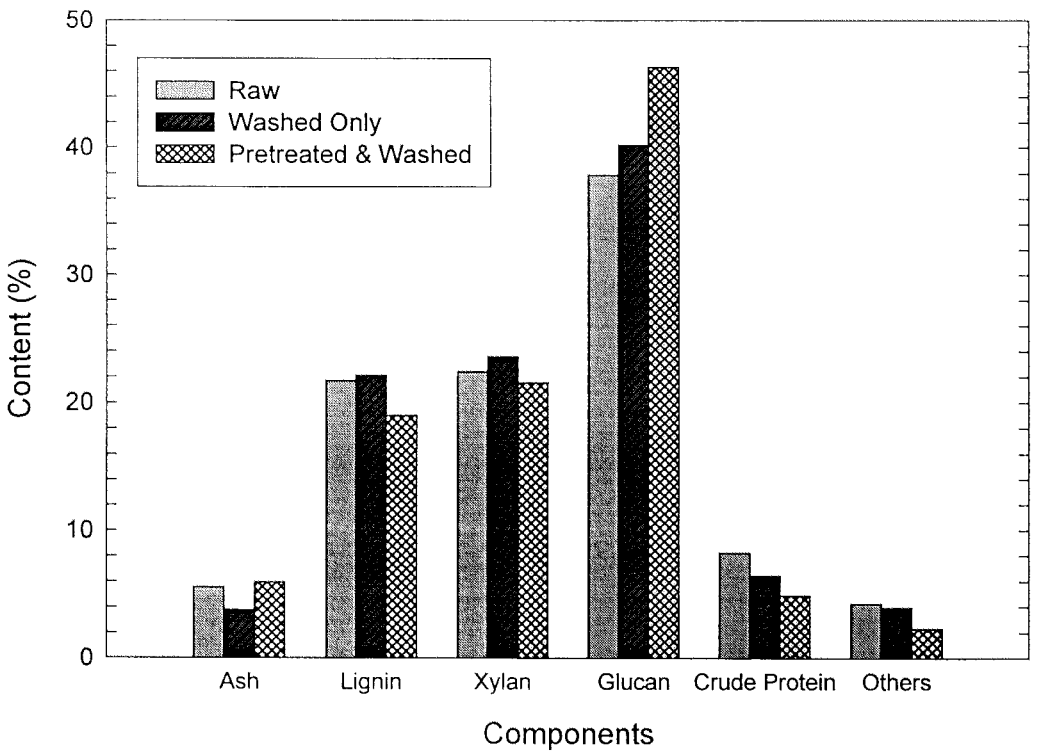


Figure 8. Compositions of raw, washed only, and pretreated & washed switchgrass (Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass.)

Enzyme Loading Studies

Purpose

A cellulase loading of 5 FPU/g dry biomass had been used in our previous studies. The study of cellulase loading is to determine if there are yield benefits from loadings higher than 5 FPU/g dry biomass or if the cellulase loadings less than 5 FPU/g dry biomass are acceptable. The sugar contents of the enzymes at various enzyme loadings were also to be explored.

Materials and Methods

Filter Paper Assay

To verify the activity of the cellulase received from NREL, a filter paper assay was performed according to the NREL standard procedure No. 006. The filter paper activity of the cellulase (Cytolase CL enzyme, Lot No. 17-92262-09) was 91.0 FPU/mL. The activity reported by the manufacturer (Environmental BioTechnologies, Inc.) was 95.3 FPU/mL.

Determination of Sugar Contents of the Enzymes

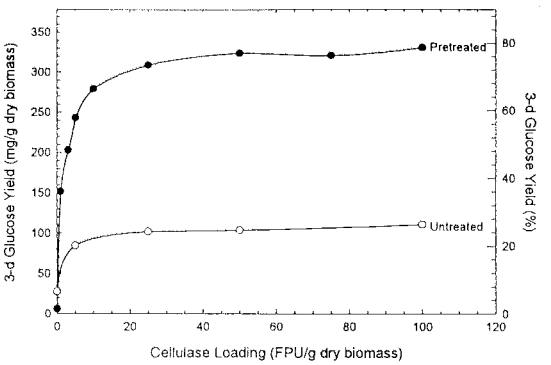
Various amounts of cellulase (10 and 20 FPU/g dry biomass; based on 7.5 g dry biomass) and cellobiase (14.2, 28.4, 56.8, and 113.6 CBU/g dry biomass; based on 7.5 g dry biomass) were incubated at 50°C, pH 4.8 for 3 days, as explained in Appendix C, "General Enzymatic Hydrolysis Procedure," except that no biomass was involved. The reducing sugar yields were determined by the DNS assay; the glucose and xylose yields were determined using HPLC (Appendix E).

Study of Cellulase Loading

Approximately 90 g (105°C dry weight) of switchgrass was pretreated under the optimal conditions (i.e., time = 2 h, temperature = 120°C, lime loading = 0.1 g/g dry biomass, water loading = 9 mL/g dry biomass, particle size = 40 mesh and finer). The pretreated and untreated switchgrass was hydrolyzed simultaneously at 50°C, pH 4.8 for 3 days, using an excess cellobiase loading (i.e., 28.4 CBU/g dry biomass) and various cellulase loadings (i.e., 0, 1, 3, 5, 10, 25, 50, 75, 100 FPU/g dry biomass). The rotating speed of the air shaker was 100 rpm and the concentration of the biomass slurry was 5%. The 3-day glucose yields were measured using YSI and HPLC, whereas the 3-day xylose yields were determined by HPLC alone (see Appendix E for step-by-step procedures).

Results and Discussions

The 3-day glucose, xylose, total sugar (i.e., glucose plus xylose), and reducing sugar yields of untreated and optimally pretreated switchgrass are plotted against cellulase loadings, as shown in Fig. 9. (Note: In general, total sugar yield is defined as the summation of glucose, xylose, and cellobiose yields. At cellulase loadings larger than 5 FPU/g dry biomass, however, the 3-d cellobiose yields were approximately zero, i.e., no peak for cellobiose was observed on the HPLC chromatograms. Hence, in enzyme loading studies, the total sugar yields only counted in glucose and xylose yields.) The experiment was performed twice. In the first run, reducing sugars and glucose were measured using the DNS assay and YSI, respectively, whereas in the second run, both glucose and xylose were measured using HPLC. The glucose yields showed good agreement with different methods of measurements; however, the reducing sugar yields were higher than the total sugar yields. This may result from alkaline oxidative degradation which converts glucose to D-glucoisosaccharinate which may be detected



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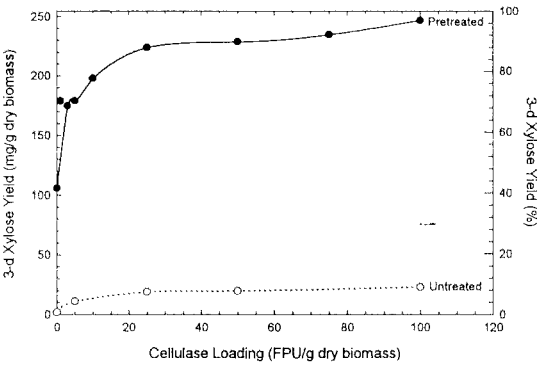

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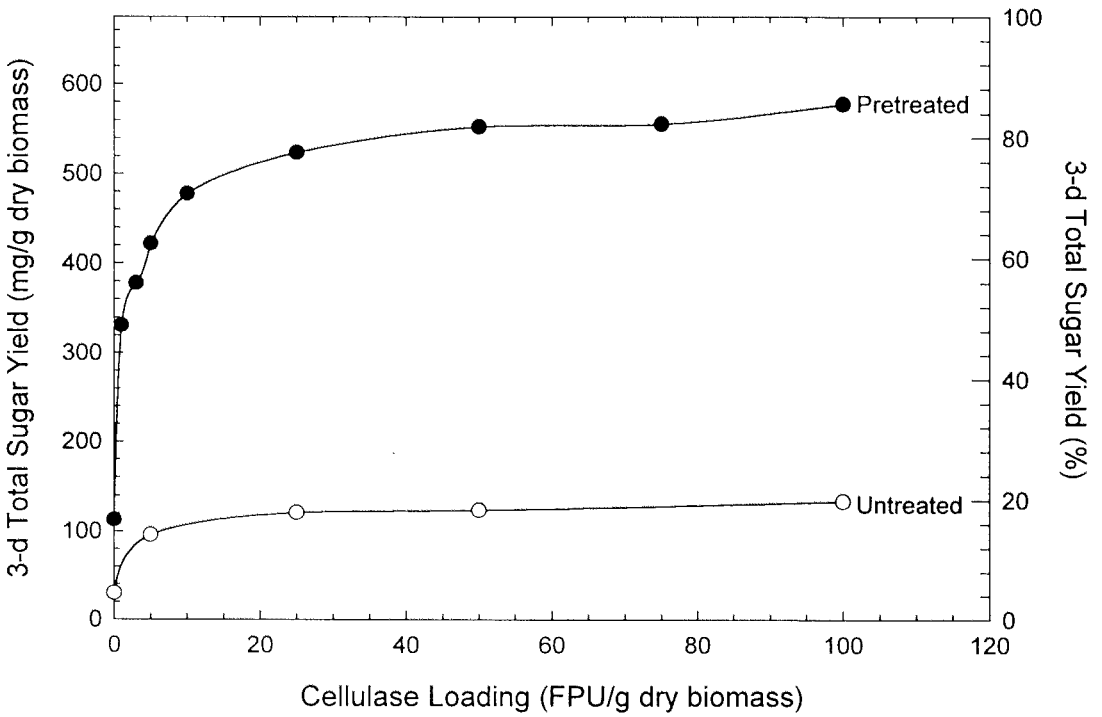


(c)

Figure 9. Sugar yields for various cellulase loadings: (a) Glucose (b) Xylose (c) Total sugar and reducing sugar (Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass)

by the DNS reagent (Holtzapple, 1981). This hypothesis will be discussed in "Oxygen-Free Lime Pretreatment."

For cellulase loadings larger than 25 FPU/g dry biomass, the sugar yields remain essentially constant. The data indicate that, at a cellulase loading of 25 FPU/g dry biomass, the cellulose sites are likely saturated by the enzyme. The maximal total sugar yield of 85% may result from carbohydrate destruction due to high-temperature lime pretreatment. A cellulase loading of 5 FPU/g dry biomass seems to be optimal from a practical viewpoint since it represents the "shoulder" of the curve. Although a cellulase loading of 10 FPU/g dry biomass gives about 12% of increase in total sugar yield, the 200% increase in enzyme usage probably cannot be justified economically. In Fig. 9, the glucose and xylose yields presented on the right axis were calculated based on switchgrass compositions reported in "Material Balances."

The sugar yields of untreated switchgrass are enormously lower than those for optimally pretreated switchgrass. This indicates that lime pretreatment causes a dramatic increase in biomass digestibility. The 3-day glucose yield of pretreated switchgrass increases about 3 times, the 3-day xylose yield increases about 11 times, and the 3-day total sugar yield increases about 4.3 times.

Fig. 10a and 10b show the 3-day reducing sugar yields and 3-day glucose yields resulting from the enzyme alone, respectively. Most of the sugar came from cellobiase. No xylose was detected. At our standard enzyme loadings (i.e., 5 FPU cellulase/g dry biomass plus 28.5 CBU cellobiase/g dry biomass) in a general 3-d enzymatic hydrolysis, the reducing sugar yield resulting from the enzymes is 4.2 mg eq. glucose/g dry biomass, and the glucose yield is 2.64 mg/g dry biomass.

Conclusions

A cellulase loading of 5 FPU/g dry biomass is selected as the optimal enzyme loading.

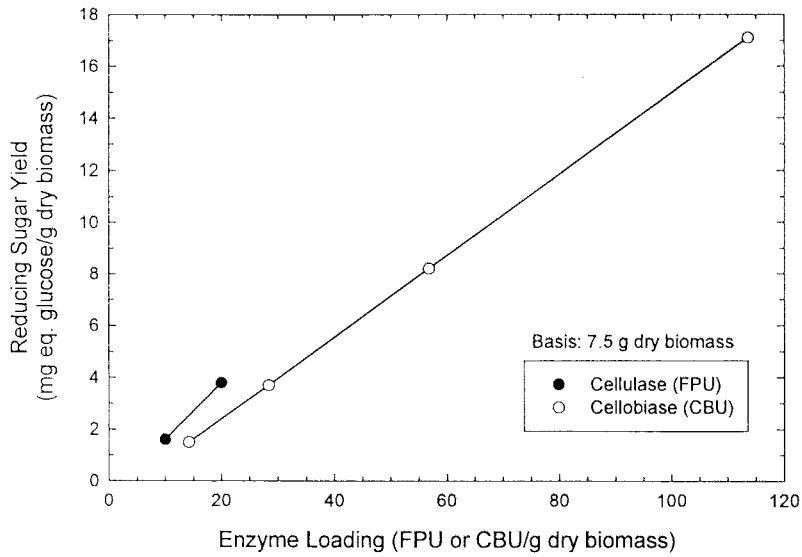
Enzymatic Hydrolysis Profiles

Purpose

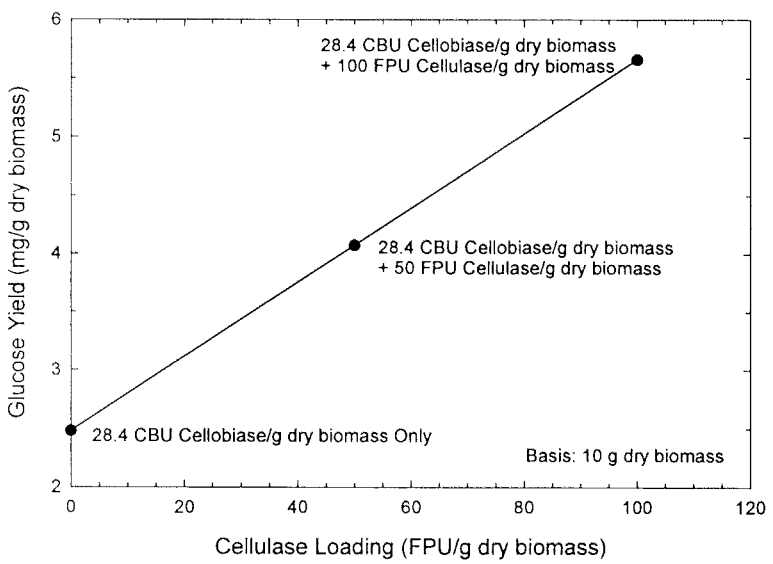
Instead of measuring just the 3-day reducing sugar yields, a complete hydrolysis profile is measured so the sugar yields are determined as a function of time.

Materials and Methods

Switchgrass (40 mesh and finer) was pretreated at 100 or 120°C for 2 hours in the presence of 0.1 g lime/g dry biomass and 9 mL water/g dry biomass. The pretreated switchgrass was then transferred from the reactors to Erlenmeyer flasks for enzymatic hydrolysis. The hydrolysis conditions were: temperature = 50°C, pH = 4.8, cellulase loading = 5 FPU/g dry biomass, cellobiase loading = 28.4 CBU/g dry biomass, rotating speed = 100 rpm. Samples were withdrawn as a function of time (i.e., 0, 1, 3, 6, 10, 16, 24, 36, 48, 72 h). Reducing sugars were measured using the DNS assay; glucose was measured using YSI and HPLC; xylose and cellobiose were measured using HPLC. This experiment was performed in triplicate. See Appendices B, C, and E for procedures of lime pretreatment, enzymatic hydrolysis, and sugar measurements, respectively.



(a)



(b)

Figure 10. 3-d sugar yields resulting from the enzymes alone: (a) Reducing sugar yields (b) Glucose yields.

Results and Discussions

Figures 11 and 12 show the yields of reducing sugars, glucose, xylose and "total sugar" (glucose + xylose) at different hydrolysis times. Again, the total sugar yields are unexpectedly smaller than the reducing sugar yields. This may be caused by alkaline oxidative degradation which converts glucose to D-glucoisosaccharinate which may be recognized by the DNS reagent (Holtzapple, 1981). This hypothesis will be studied in next section, "Oxygen-Free Lime Pretreatment."

The sugar yields of pretreated switch grass are significantly higher than untreated switch grass. The 3-day reducing sugar yield of pretreated switch grass increases about 5 times (i.e., from 102 to 538 mg eq. glucose/g dry biomass), the 3-day total sugar yield increases about 7 times (i.e., from 8.7% to 58.1%), the 3-day glucose yield increases about 5 times (i.e., from 12.3% to 58.0%), and the 3-day xylose yield increases about 21 times (i.e., from 2.8% to 58.1%). As shown in Fig. 11, hydrolysis does not proceed significantly further after 24 h.

Oxygen-Free Lime Pretreatment

Purpose

The hydrolysis profile studies show that the total sugar yields (measured by HPLC) are significantly smaller than the reducing sugar yields (measured by the DNS assay). It is suspected that, during lime pretreatment, alkaline oxidative degradation converts glucose to D-glucoisosaccharinate which may be recognized by the DNS reagent (Holtzapple, 1981). Therefore, an oxygen-free lime pretreatment was conducted to determine if the oxidative degradation of glucose causes the differences between the results of DNS assay and HPLC measurements.

Materials and Methods

The pretreatment was conducted in an oxygen-free environment. After measured amounts of switchgrass, lime, and water were loaded, the reactors were evacuated and then filled with nitrogen. Nitrogen served as an inert gas which kept the pressure inside the reactors constant (ca. 1 atm). The optimal pretreatment conditions were used, i.e., 120°C, 2 h, 0.1 g lime/g dry biomass, and 9 mL water/g dry biomass. The oxygen-free pretreated switchgrass, as well as untreated switchgrass, normally pretreated switchgrass, and α-cellulose standard, were simultaneously hydrolyzed using the NREL standard procedure No. 009. The glucose yields were measured using HPLC. This experiment was performed in duplicate except for the untreated sample.

Results and Discussions

Table 9 shows the 3-day and 7-day glucose yields of α-cellulose, untreated, prtreated, and oxygen-free pretreated switchgrass. No noticable difference in glucose yields between normal and oxygen-free pretreatments was observed.

Conclusions

The data do not prove our hypothesis. The discrepancy between the reducing sugar measurements and the total sugar measurements (i.e., glucose + xylose measured by HPLC) probably results because of inaccuracies associated with expressing xylose as equivalent glucose.

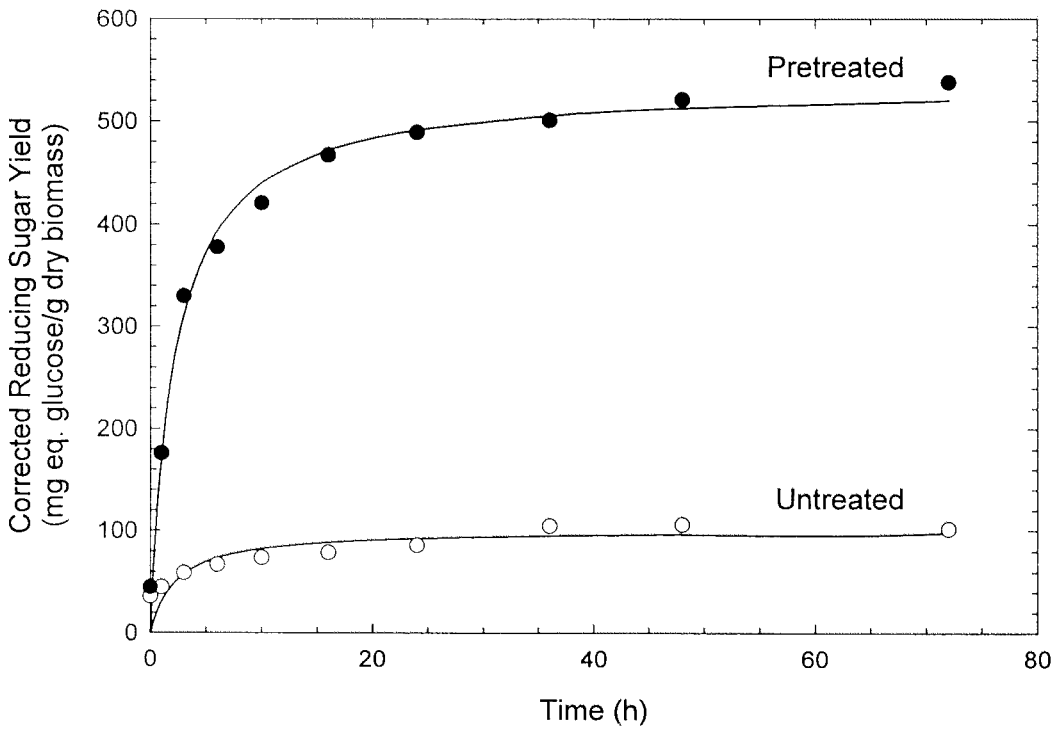
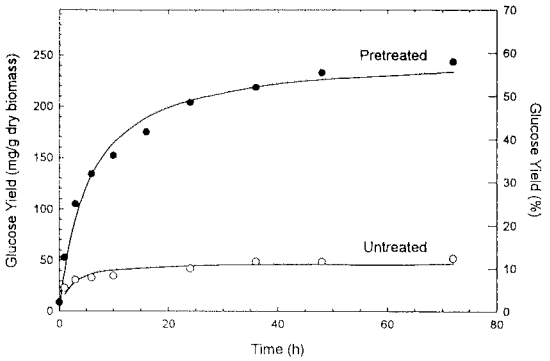


Figure 11. 3-d hydrolysis profiles of switchgrass: reducing sugar.
(Pretreatment conditions: 100°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)



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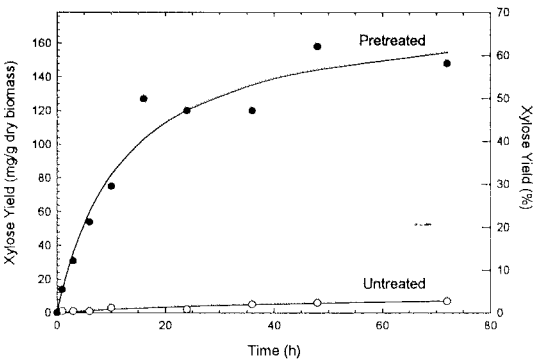

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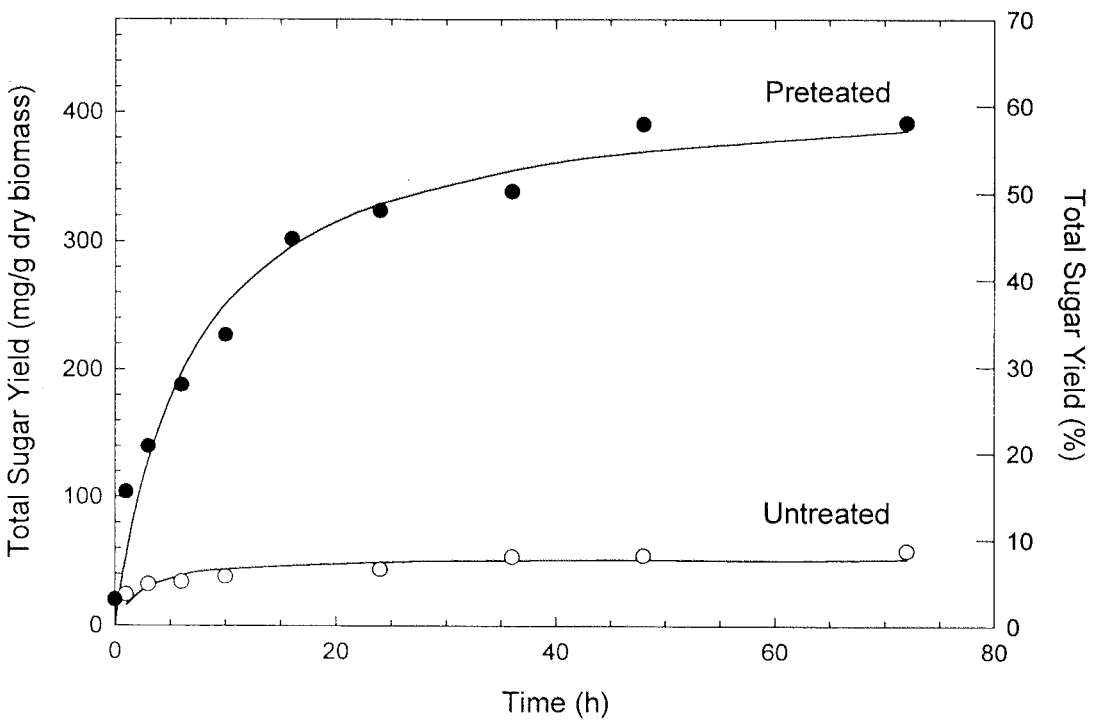

(c)

Figure 12. 3-d hydrolysis profiles of switchgrass: (a) glucose (b) xylose (c) total sugar. (Pretreatment conditions: 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass)

Table 9. Enzymatic Saccharification of Switchgrass (NREL Procedure No. 009)

Substrate	3-day Glucose Yield			7-day Glucose Yield		
	Run 1	Run 2	Average	Run 1	Run 2	Average
α-cellulose	102.85%	100.94%	101.90%	107.49%	99.07%	103.28%
Untreated Switchgrass	18.09%	-----	18.09%	20.64%	-----	20.64%
Pretreated Switchgrass	67.06%	68.07%	67.57%	73.07%	76.86%	74.96%
Deoxygenic Pretreated Switchgrass	67.57%	68.14%	67.86%	74.07%	73.94%	74.00%

Lime Recovery

Purpose

In the industrial process, lime will be neutralized with carbon dioxide. The pretreated biomass will be washed with water to remove the lime. The wash water will be contacted with carbon dioxide to precipitate calcium carbonate. The calcium carbonate may be separated from the liquid and converted to lime. This process allows lime to be recycled so that the cost of lime consumption is effectively minimized. This study is to determine how much lime can be recovered after switchgrass is optimally pretreated.

Materials and Methods

A large amount of switchgrass (60 g) was optimally pretreated (i.e., 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass). The biomass mixture was filtered and the filtrate was transferred to a 500-mL graduated cylinder. The wet biomass was squeezed and the squeezed liquid was collected and mixed with the previous filtrate in the 500-mL cylinder. The total filtrate volume was measured (ca. 490 mL). The squeezed biomass was stored in a 600-mL beaker for further washing. Half of the filtrate (ca. 245 mL) was contacted with carbon dioxide while the pH was monitored. A small amount of sample (ca. 10 mL) was withdrawn and the calcium content was measured as a function of the pH. The pH (ca. 7.5) with the minimal calcium concentration was used as the optimal pH for further precipitation. The other half of the filtrate was contacted with carbon dioxide until the pH dropped to 7.5. A small liquid sample was withdrawn and the calcium content was measured before and after carbon dioxide contact. The squeezed biomass was mixed with fresh distilled water (ca. 100 mL) and stirred for 10 min. The biomass mixture was filtered and the wet biomass was squeezed. (For each cycle, the squeezed liquid was combined with the filtrate and the mixed liquid was then contacted with carbon dioxide until the pH reached 7.5.) The biomass was repeatedly washed with fresh distilled water for 10 times. Ten milliliters of sample was withdrawn and the calcium content was measured before and after carbonation. The carbonated liquid sample of each cycle was filtered through a 0.22-μm nylon filter which had been dried at 105°C and whose dry weight had been recorded. The filters as well as the filter cakes (i.e., calcium carbonate) were dried at 105°C and the dry weights were measured. The calcium carbonate precipitate was collected for carbohydrate analysis, as described in the NREL standard procedure No. 002. The calcium content of the samples were measure using atomic adsorption (see Appendix F).

Results and Discussions

Fig. 13a shows the calcium content of the first half of the filtrate as a function of the pH. The optimal pH for carbonation was 7.5. Fig 13b shows the calcium content in the wash water before and after carbonation. The two curves coincide indicating that the effectiveness of lime recovery for pretreated switchgrass is fairly low. After 5 washings, no more lime was removed from the pretreated biomass. This verifies the washing procedure for the acid study in which the biomass was washed 10 times to remove the lime. There was only 0.18 g of calcium carbonate precipitate collected after 10 washings and carbonations. The carbohydrate analysis showed that there was essentially no carbohydrate in the precipitate.

Table 10 shows the calcium material balance during lime recovery. After pretreatment, 62% of the added calcium was in the biomass and 38% was in the filtrate. The filtrate was carbonated allowing 4% of the added calcium to be recovered as a calcium carbonate precipitate. After repeated washings, more calcium was removed from the biomass so that only 31% of the added calcium remained in the biomass; the remaining calcium was primarily in the liquid phase since very little additional calcium could be recovered by carbonating the wash water.

The residual calcium in the biomass was 0.025 g calcium/g dry pretreated biomass[1]. According to Maiorella (1983), in alcoholic yeast fermentations, the following amounts of organic acids result from the fermentation of sugars: 0.0023 g acetic acid/g sugar, 0.0016 g butyric acid/g sugar, 0.00107 g formic acid/g sugar, 0.00190 g lactic acid/g sugar, and 0.00171 g succinic acid/g sugar. Calculations show (see Appendix I) that these acids will consume less than 10% of the residual calcium in the pretreated biomass. The remaining calcium will either remain complexed with unreacted biomass (e.g., lignin) or will precipitate as calcium bicarbonate provided the saccharification/fermentation pH is 5.5 or greater. If the saccharification/fermentation pH is less than 5.5, sulfuric acid (or another strong acid) must be added which will react with the calcium bicarbonate to form calcium sulfate (gypsum). The calcium cannot be recovered from gypsum, so it would have to be disposed of as a waste product. If the saccharification/fermentation is operated below pH 5.5, the amount of gypsum that will be produced is about 0.057 g gypsum/g raw biomass[2]. Given a lime cost of $57.2/tonne and a sulfuric acid cost of $75/tonne, the chemical consumption costs $4.42/tonne raw biomass[3]. Additional costs would be incurred for gypsum disposal. Obviously, there are great incentives for avoiding these costs either by completely washing the calcium out of the biomass (if possible), or operating the saccharification/fermentation above pH 5.5.

Conclusions

Fig. 14 shows four possible processes in which lime is used as the pretreatment agent.

In Process A, the lime-treated biomass is thoroughly washed with water which is subsequently carbonated to precipitate calcium carbonate. The calcium carbonate is converted to quicklime (CaO) in a lime kiln.

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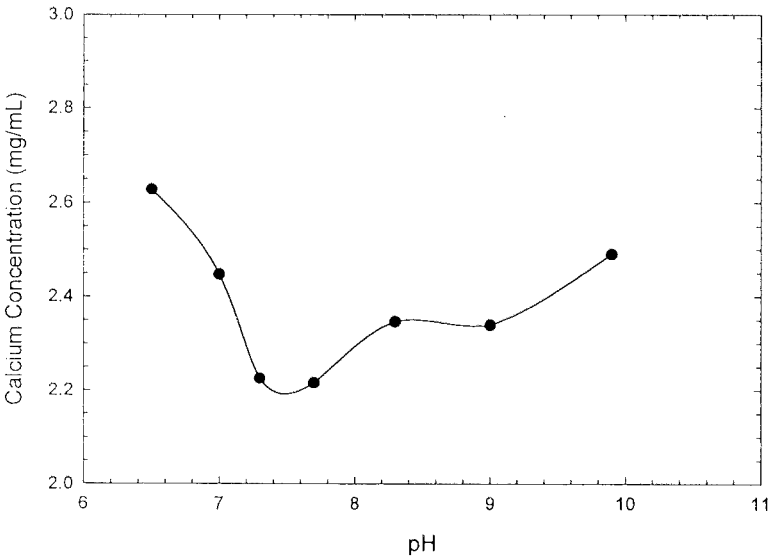
1 (1.02 g Ca/60 g dry raw biomass) × (1 g dry raw biomass/0.6761 g pretreated biomass) = 0.025 g Ca/g pretreated biomass

2 (0.0025 g calcium/g dry pretreated biomass) × (136 g $CaSO_4$/40g Ca) × (0.6761 g dry pretreated biomass/ g dry raw biomass) = 0.057 g $CaSO_4$/g raw biomass

3 (0.057 tonne gypsum/tonne raw biomass) × (40 tonne Ca/136 tonne $CaSO_4$) × (56 tonne CaO/40 tonne Ca) × ($57.2/tonne CaO) = $1.34/tonne raw biomass
(0.057 tonne gypsum/tonne raw biomass) × (98 tonne H_2SO_4/136 tonne $CaSO_4$) × ($75/tonne H_2SO_4) = $3.08/tonne raw biomass



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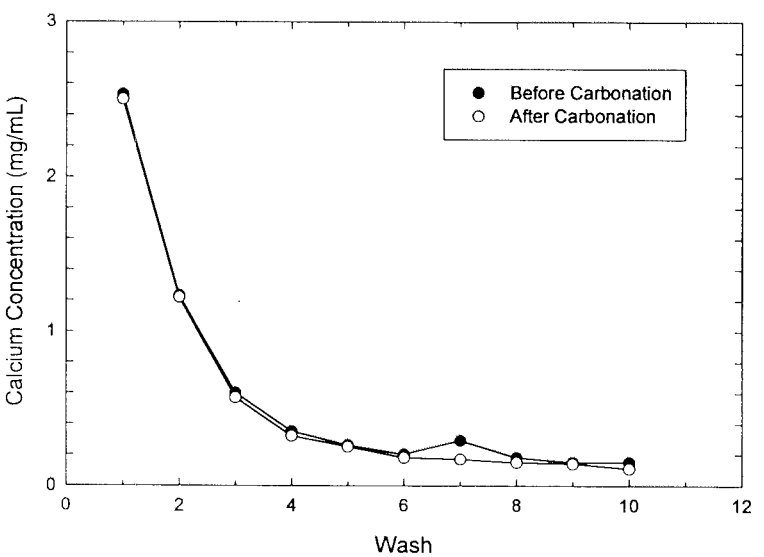
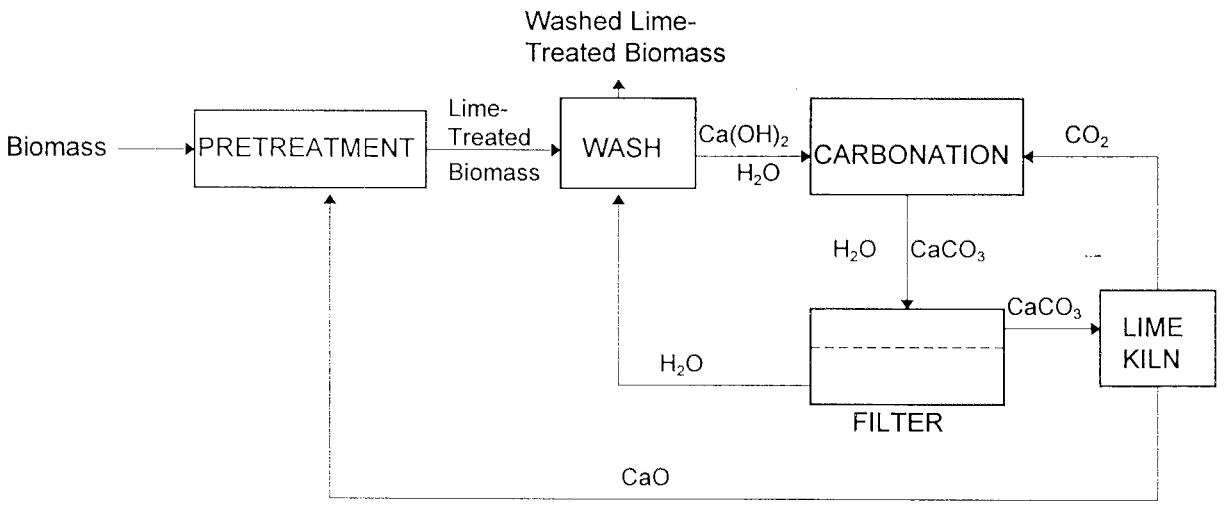
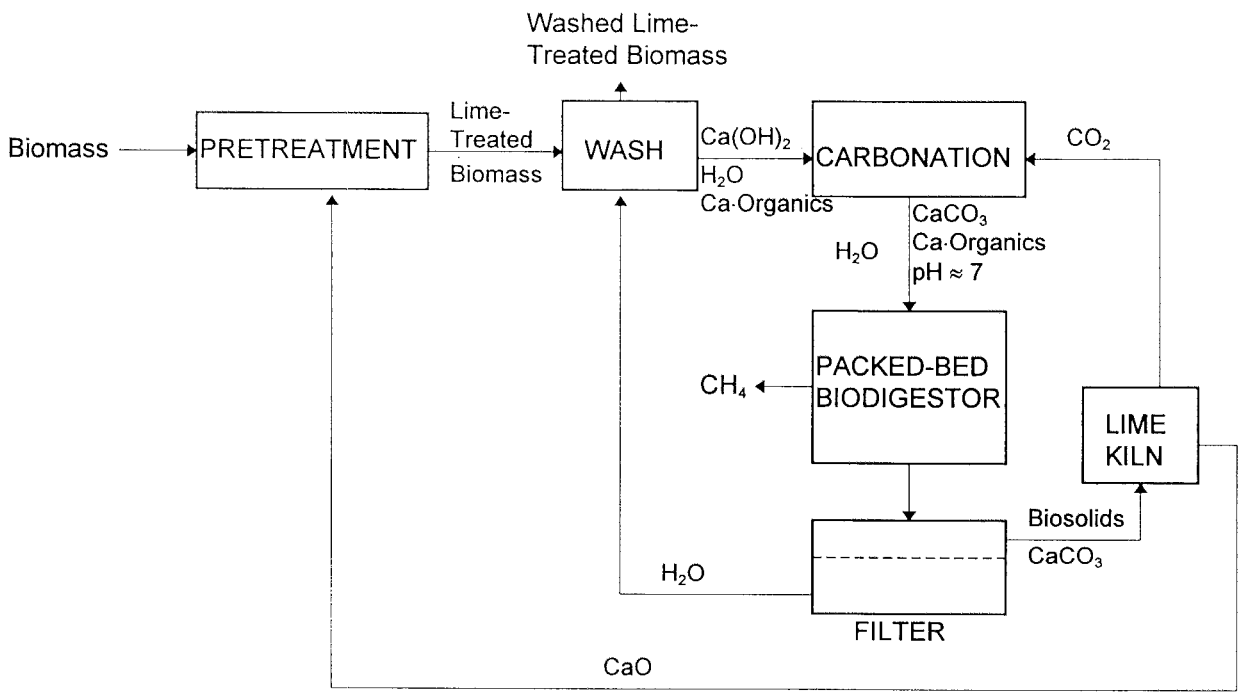


(b)

Figure 13. (a) Optimization of the pH (b) Lime recovery (Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass)

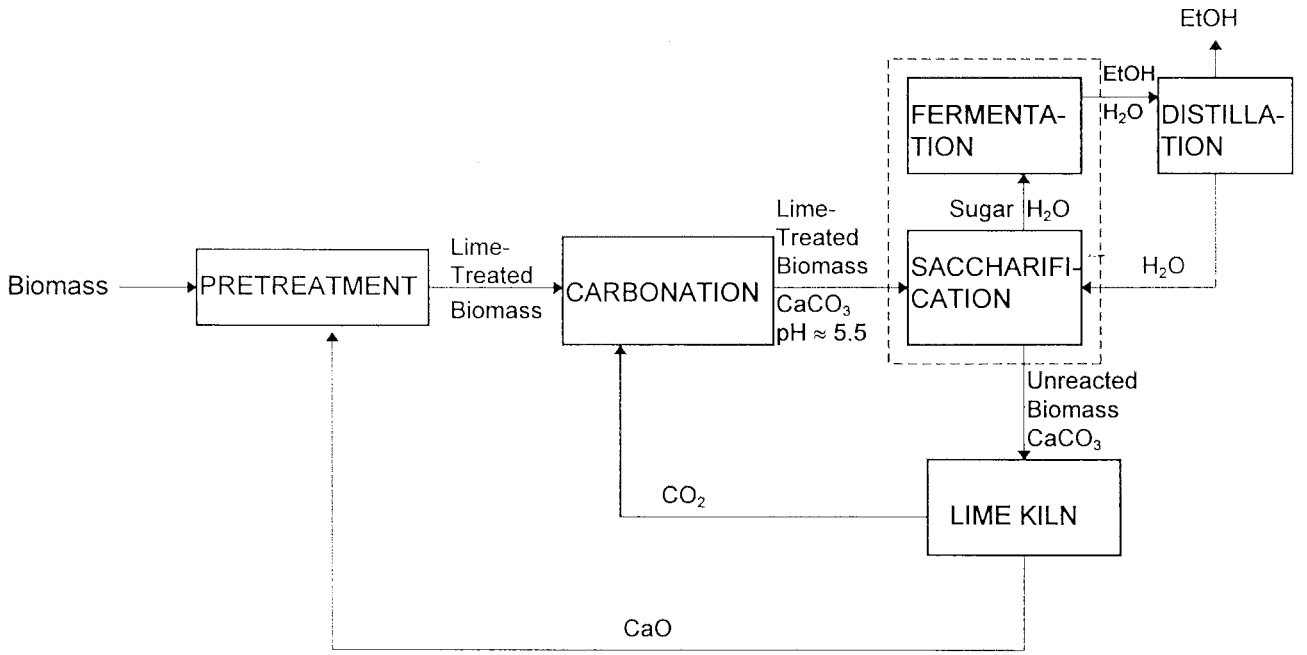


PROCESS A

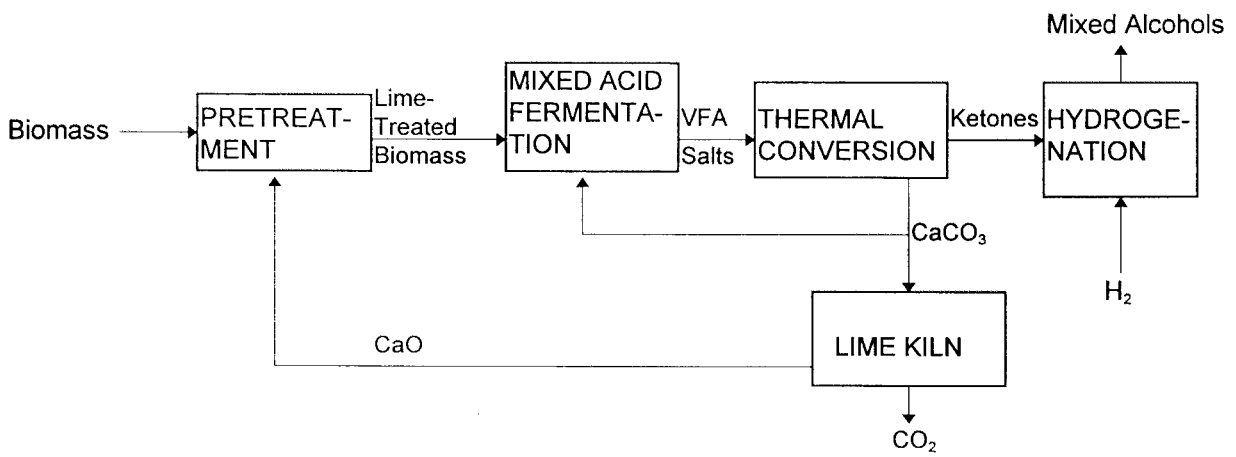


PROCESS B

Figure 14. Four process alternatives that incorporate lime pretreatment



PROCESS C



PROCESS D

Figure 14. (Continued) Four process alternatives that incorporate lime pretreatment.

Table 10 . Material Balance of Calcium during Lime Recovery for Switchgrass

Calcium Input	3.24 g (= 6.0 g calcium hydroxide)[a]		
	Biomass	Liquid Phase	Calcium Carbonate Precipitate
Before Pretreatment	0 g[b]	0 g	-----
After Pretreatment	2.02 g (62%)	1.22 g[c] (38%)	-----
After Carbonation of Filtrate	2.02 g (62%)	1.09 g[c] (34%)	0.13 g (4 %)
After Repeated Washings and Carbonations	1.02 g (31%)	2.09 g[c] (63%)	0.19 g (6%)
a. Added to 60 g dry raw biomass.			
b. Added calcium only.			
c. Measured by atomic adsorption.			

In Process B, the lime-treated biomass is thoroughly washed with water. The wash water is contacted with carbon dioxide to adjust the pH near neutrality. Then the water is sent to a packed-bed biodigestor where organic matter is fermented to methane and carbon dioxide. The carbon dioxide reacts with the calcium to form calcium carbonate which attaches to the biofilm on the packed-bed. As the biofilm sluffs off, it is collected and sent to the lime kiln. The biosolids provide some process heat for the endothermic reaction of calcium carbonate to quicklime (CaO).

In Process C, the lime-treated biomass is directly carbonated to neutralize the lime to calcium carbonate. Because carbon dioxide is not a strong acid, the minimum pH is about 6.0. The lime-treated, neutralized biomass would then be sent directly to the saccharification vessel for hydrolysis of the carbohydrate polymers to soluble sugars. These soluble sugars may be converted directly to alcohols in the saccharification vessel (i.e., simultaneous saccharification/fermentation) or the fermentation may occur in a separate vessel. Unreacted solids (lignin, recalcitrant carbohydrate) and calcium carbonate precipitate will be sent to a lime kiln; the unreacted solids will provide process heat for the endothermic reaction of calcium carbonate to quicklime (CaO).

In Process D, the lime-treated biomass is directly fed to a fermentor in which a mixed culture of acid-forming microorganisms directly digests the biomass to volatile fatty acids (e.g., acetate, propionic, and butyric acids). The volatile fatty acids (VFA) immediately react with the lime to form VFA salts (e.g., calcium acetate, propionate, and butyrate). The VFA salts solution exiting the fermentor is dewatered and then thermally converted to ketones (e.g., acetone, methyl ethyl ketone, diethyl ketone, methyl propyl ketone). The ketones may be used directly for fuel or hydrogenated to form higher alcohols (e.g., isopropanol, isobutanol, isopentanol).

Table 11 compares the characteristics of each process alternatives described in Fig. 14. In the case of switchgrass, the conditions required by Process A cannot be met; too little $CaCO_3$ is precipitated after carbonation and too much biomass is solublized by the pretreatment. (In our previous studies with bagasse (Nagwani, 1992), the conditions required by Process A have been met.) However, the conditions for Process B, C, and D can be met by switchgrass. The choice of process depends primarily upon the desired product; Process B produces ethanol and methane, Process C produces ethanol only, and Process D produces mixed alcohols. Process C requires that the cellulase operate above pH 5.5, the lowest pH

achievable by carbon dioxide. (If a stronger acid, such as sulfuric acid were added, the calcium would react to calcium sulfate (gypsum) from which the calcium is unrecoverable.)

Table 11. Comparison of Process Alternatives

Processes	$CaCO_3$ precipitation after carbonating wash water	Removal of calcium from biomass during washings	Solubilization of organic matter from lime pretreatment	pH optimum of extracellular cellulase	Biomass product
Process A	High	High	Low	2 — 11	EtOH
Process B	Low	High	High	2 — 11	EtOH & CH_4
Process C	Irrelevant	Irrelevant	Irrelevant	> 5.5	EtOH
Process D	Irrelevant	Irrelevant	Irrelevant	Irrelevant	Mixed Alcohols

Simultaneous Saccharification/Fermentation (SSF)

Purpose

This study is to determine the initial hydrolysis rates and the ethanol yields from pretreated switchgrass under simultaneous saccharification/fermentation conditions.

Materials and Methods

Determination of Initial Hydrolysis Rate

Switchgrass was pretreated at the optimal conditions (i.e., 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass) and then hydrolyzed at 37°C, pH 5.0 for 24 hours with an enzyme loading of 25 FPU cellulase/g cellulose. The concentrations of glucose, xylose, and cellobiose were measured using HPLC as a function of time (i.e., 0, 1, 3, 6, 12, 24 h). The glucose and xylose yields were calculated using the following formulas:

$$\text{Glucose Yield} = \frac{[\text{Glucose}] + 1.053\,[\text{Cellobiose}]}{1.111 f_G\,[\text{Biomass}]} \times 100 \qquad (2)$$

$$\text{Xylose Yield} = \frac{[\text{Xylose}]}{1.136 f_X\,[\text{Biomass}]} \times 100 \qquad (3)$$

where [Glucose] = glucose concentration (mg/mL)
[Cellobiose] = cellobiose concentration (mg/mL)
[Xylose] = xylose concentration (mg/mL)
[Biomass] = dry biomass concentration in the beginning of the hydrolysis (mg/mL)
f_G = glucan fraction in dry biomass
f_X = xylan fraction in dry biomass

The NREL standard procedure No. 008 was exactly followed except that the recipe was scaled up by 15 times since it was not possible to pretreat such a small quantity of biomass (ca. 0.3 g). This experiment was performed in triplicate.

Simultaneous Saccharification/Fermentation

Switchgrass was optimally pretreated as in "Determination of Initial Hydrolysis Rate." The pretreated switchgrass was transferred to Erlenmeyer flasks equipped with water traps for SSF. The cellulase loading was 25 FPU/g cellulose and the concentration of the yeast inoculum was 10% v/v (e.g., 20 mL of yeast inoculum was added if the total working volume was 200 mL). The concentrations of glucose, cellobiose, and ethanol were measured as a function of time (i.e., 24, 48, 72, 96, 120, 144, and 168 h). The glucose and cellobiose were measured using HPLC whereas the ethanol content was measured using GC. Appendix G explains the procedure for ethanol measurements. The ethanol yield is calculated using the following formula:

$$\text{Ethanol Yield} = \frac{[\text{EtOH}]-[\text{EtOH}]_i}{0.568 f_G\,[\text{Biomass}]} \times 100 \qquad (4)$$

where

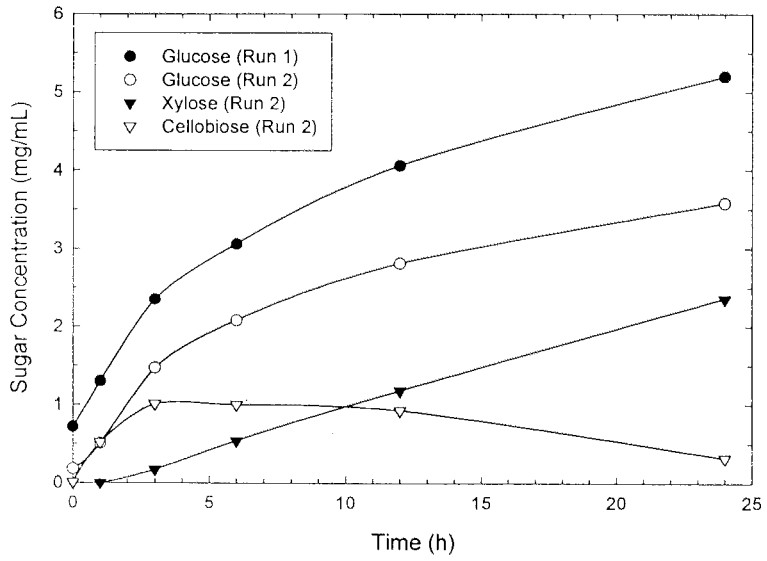
$[\text{EtOH}]_i$ = Ethanol concentration at the beginning of SSF (g/L)
[EtOH] = Ethanol concentration at a time point (g/L)
[Biomass] = dry biomass concentration at the beginning of SSF (g/L)
f_G = glucan fraction in dry biomass

The experiment was repeated twice and the NREL standard procedure No. 008 was exactly followed except that, in the first run, excess water (40 mL per 100 mL sample) was evaporated from the pretreated biomass slurry in a freeze drier since there was more water in the slurry after pretreatment than is allowed in the recipe; in the second run, the initial biomass concentration was scaled down from 3% of cellulose to 1.9% and the total working volume was doubled (i.e., 200 mL). The change in the procedure for the second run resulted because, to pretreat such a large amount of biomass, the required water loading plus the rinse water (for transferring the biomass from the reactors to the flasks) would exceed the allowed total working volume. Also, in the second run, antibiotics (penicillin and streptomycin) were added to the biomass mixtures, as allowed in the procedure. This experiment was performed in duplicate.

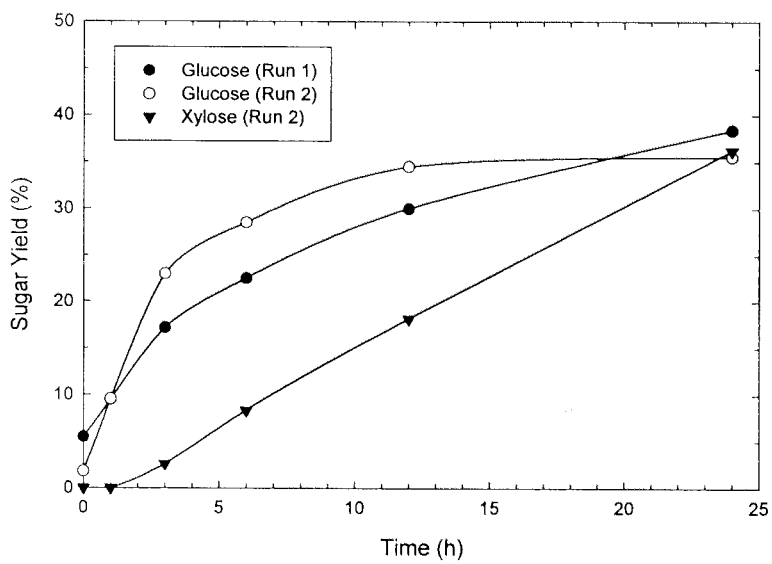
Results and Discussions

Determination of Initial Hydrolysis Rate

Fig. 15a shows the concentrations of glucose, xylose, and cellobiose at different hydrolysis times, whereas Fig. 15b shows the glucose and xylose yields as a percentage of the theoretical yields during hydrolysis for two independent runs. The glucose was measured using YSI in the first run whereas HPLC was used in the second run. The glucose yields in both runs agreed well; however, the glucose concentrations obtained from the first run were higher than those from the second run. The discrepancy resulted from different initial biomass concentrations (i.e., 3.2% and 2.6% for Run 1 and Run 2, respectively). The hydrolysis rate of cellulose (i.e., conversion of cellulose to cellobiose and glucose) was fast in the beginning of the hydrolysis, but started declining after 5 h. The hydrolysis rate of hemicellulose (i.e., conversion of hemicellulose to primarily xylose) was slower and essentially constant during the hydrolysis period. The glucose yield at 24 h was 36% and the xylose yield was 35%. The final pH of each sample was about 4.3 in both runs and afterwards, no contamination was observed on the YPD plates.



(a)



(b)

Figure 15. Initial hydrolysis rate study under SSF: (a) Sugar concentrations (b) Sugar yields
(Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass)

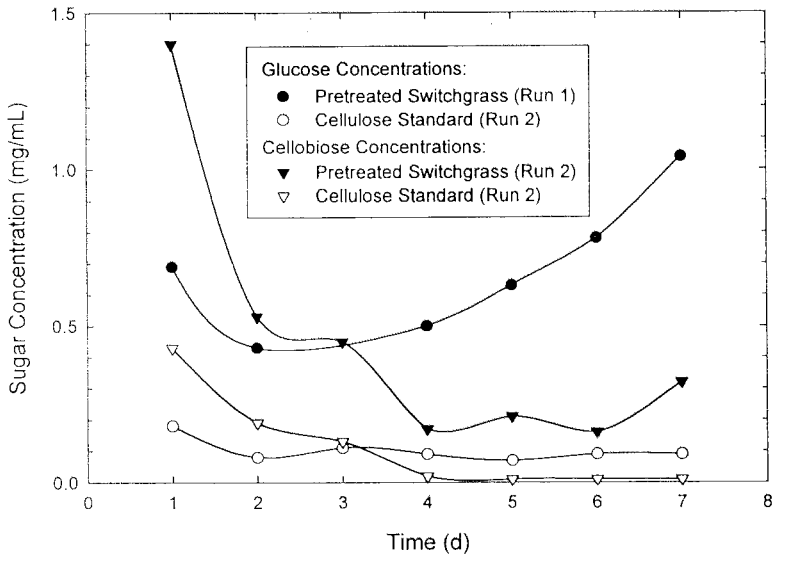
Simultaneous Saccharification/Fermentation

Fig 16a shows the glucose and cellobiose concentrations during SSF whereas Fig 16b shows the ethanol yields. For the pretreated switchgrass sample, the glucose concentration dropped initially and the glucose started accumulating after 60 h, whereas the cellobiose concentration declined steeply and then essentially remained constant after 96 h. The glucose and cellobiose concentrations of the α-cellulose sample were much lower and more steady than for the switchgrass sample. As shown in Fig. 16b, the pretreated switchgrass did not produce ethanol significantly further after 48 h. The ultimate ethanol yield of pretreated switchgrass was about 70% of the theoretical yield, whereas about 90% of ethanol yield was obtained from the α-cellulose standard. The yeast culture before inoculation had a dry cell weight of 0.0063 g/mL, an optical density at 600 nm of 2.265 under a dilution factor of 3, a glucose concentration of 0.14 g/L, and an ethanol concentration of 18.30 g/L. The pH readings after 7 days of SSF were 4.18 and 4.12 for the two switchgrass samples and 4.45 and 4.30 for the two α-cellulose standards. After streaking samples on YPD plates and incubating them at 38°C for 24 h, no contamination was observed.

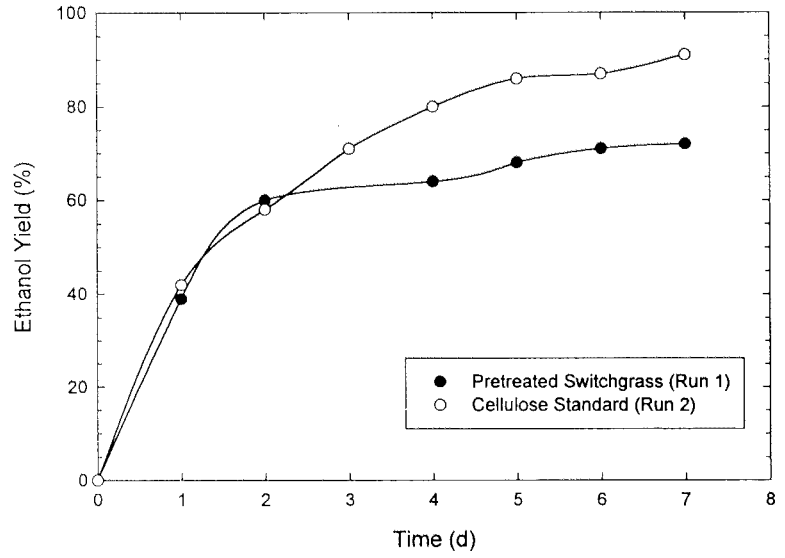
Conclusions

The glucose yield can be increased from 20% to 63% and the xylose yield from 4% to 70% by lime pretreatment, using 5 FPU cellulase/g dry biomass (Fig. 9). The best ethanol yield we obtained was 70% of the theoretical yield. Lime pretreatment removes about 29% of lignin, 10% of glucan and 26% of xylan. From switchgrass, lime recovery by carbonating wash water is not high. (Our previous study has shown a high lime recovery for other biomass such as bagasse (Nagwani, 1992).) However, three process alternatives are available that overcome this limitation (see Processes B, C, and D in Fig. 14). The optimal conditions for lime pretreating switchgrass are: 100 or 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, and a particle size of 20 mesh or smaller.

The results show lime pretreatment is an excellent pretreatment method. From a technical viewpoint, the pretreatment causes high enzymatic digestibility and the simplicity of the process makes it highly applicable. Furthermore, the low cost of lime, the potential ease of lime recovery, and the mild process conditions — which substantially reduce the capital cost — make lime pretreatment a potentially economical process.



(a)



(b)

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Appendix A. Sample Preparation

Swtichgrass was used as the substrate in this study, which was provided by the National Renewable Energy Laboratory (NREL). The sample was already air-dried and clean so washing was not necessary. The material with an initial size of approximately 7 mm×400 mm was first ground by a hammer mill to reduce the particle size to approximately 3 mm×30 mm. A portion of the 3 mm×30 mm samples was then ground by another hammer mill to a finer particle size of approximately 1 mm×3 mm. The 1 mm×3 mm sample was further ground using a Thomas-Wiley laboratory mill and sieved through various kinds of sieves according to the required particle sizes. In this study, a particle size of 40 mesh and finer was used in most experiments.

Appendix B. Lime Pretreatment

Lignocellulosic substrate was pretreated with lime (calcium hydroxide) in the presence of water. Six 1.5 in I.D. × 5 in long, 304 stainless steel capped pipe nipples were used as the pretreatment reactors. To ensure thorough mixing of contents inside, the pipe nipples were mounted on a rotating shaft inside an oven providing the desired pretreatment temperature.

To perform the pretreatment, the oven had to be heated in advance to reach the desired temperature. Ground switchgrass (7.5 g dry weight) and calcium hydroxide (according to the desired lime loading) were placed in each reactor (with the end caps closed) and thoroughly mixed using a spatula. Distilled water (according to the desired water loading) was then added to the dry mixture. After tightly capping the reactors, they were placed in boiling water for 10 min to quickly reach a high temperature and then put into the oven. The motor was turned on to start the rotating shaft and the reactors were left for the desired pretreatment time. After the pretreatment time elapsed, the reactors were moved out of the oven and immersed in cold water to cool them to ambient temperature. Samples were then removed for enzymatic hydrolysis.

General Lime Pretreatment Procedure

1. Heat the oven to the desired pretreatment temperature. The oven takes about two to three hours to reach a stable temperature. Keep the rotating device inside the oven during heating so that it gets prewarmed.
2. Remove the old Teflon tape and clean the threads at the open end. Wrap clockwise at least four layers of fresh Teflon tape.
3. Label and number all the reactors. Two or four or six reactors can be run each time.
4. Weigh ground and sieved (40 mesh) material that has 7.5 g dry weight. Using a funnel, pour it in the labeled reactors.
5. Weigh calcium hydroxide, $Ca(OH)_2$, according to the desired lime loading, and pour into the reactors with the biomass.
6. Using a spatula, mix $Ca(OH)_2$ and biomass thoroughly. This dry mixing is essential to ensure a uniform reaction.
7. Pour water according to the desired water loading.
8. Close the reactors by placing the cap on the open end. Hold the nipple in the vice and tighten the cap using a pipe wrench.
9. Place the reactors in boiling water for about five minutes, for a 50°C run, to about fifteen minutes, for a 130°C run. The water boiler takes about 20 minutes to heat up, so it must be turned on before hand.
10. Place the holders of the reactors in the slots of the rotating rod and tighten the set screws.
11. Place the device in the oven and couple it with the motor using the coupling arrangement.
12. Turn on the motor and keep the rotation speed at the minimum possible. Make sure that the motor does not fall and the motor does not stop rotating. Check periodically during the desired pretreatment time.
13. Observe the oven temperature.
14. After the pretreatment time has elapsed, take out the reactors and place them in a cold tap-water bath. Let them cool for about ten minutes.

Lime Pretreatment Procedure for Acid Study

1. Heat the oven to the desired pretreatment temperature (100°C). The oven takes about two to three hours to reach a stable temperature. Keep the rotating device inside the oven during heating so that it gets prewarmed.
2. Remove the old Teflon tape and clean the threads at the open end. Wrap clockwise at least four layers of fresh Teflon tape.
3. Label and number all the reactors. Six reactors will be used.
4. Weigh ground and sieved (40-mesh) material that has 10.0 g dry weight (a determination of moisture content of air-dried biomass has to be completed in advance, following the NREL standard procedure No. 001). Using a funnel, pour it in the labeled reactors.
5. Weigh calcium hydroxide, $Ca(OH)_2$, according to the desired lime loading (1.0 g), and pour into the reactors with the biomass.
6. Using a spatula, mix $Ca(OH)_2$ and biomass thoroughly. This dry mixing is essential to ensure a uniform reaction.
7. Pour water according to the desired water loading (100.0 mL).
8. Close the reactors by placing the cap on the open end. Hold the nipple in the vice and tighten the cap using a pipe wrench.
9. Place the reactors in boiling water for about ten minutes. The water boiler takes about 20 minutes to heat up, so it must be turned on before hand.
10. Place the holders of the reactors in the slots of the rotating rod and tighten the set screws.
11. Place the device in the oven and couple it with the motor using the coupling arrangement.
12. Turn on the motor and keep the rotation speed at the minimum possible. Make sure that the motor does not fall and the motor does not stop rotating. Check periodically during the desired pretreatment time.
13. Observe the temperature of the oven.
14. After the pretreatment time (1 h) has elapsed, take out the reactors and place them in a cold tap water bath. Let them cool for about ten minutes.
15. Wash the pretreated biomass with water as explained in Appendix D, "Washing Procedure for Acid Study."

Appendix C. Enzymatic Hydrolysis

Lime-pretreated switchgrass was transferred from the reactors to Erlenmeyer flasks with distilled water. Citrate buffer (1.0 M, pH 4.8,) and sodium azide solution (0.01 g/mL) were added to the slurry to keep constant pH and prevent growth of microorganisms, respectively. Glacial acetic acid was then added to reduce the pH from about 11.5 to 4.8. Then the total volume of the slurry was made up to 150 mL by adding distilled water. The flask was placed in a 100-rpm shaking air bath. When the temperature reached 50°C, cellulase and cellobiase were added to the flask. In the optimization studies, samples were withdrawn after 3 days and then glucose and reducing sugar were measured as 3-day yields. When the hydrolysis profiles were performed, samples were withdrawn as a function of time (i.e., 0, 1, 3, 6, 10, 16, 24, 36, 48, and 72 h) and then sugars were measured at each time point. See the following for the complete hydrolysis procedures. The same procedure was also applied to untreated switchgrass.

General Enzymatic Hydrolysis Procedure

1. Prepare 250 mL 1-M citrate buffer (pH 4.8) and 100 mL 0.01-g/mL sodium azide solution. (To prepare citrate buffer, first dissolve 210 g of citric acid monohydrate in 1000 mL of distilled water, then adjust the pH to 4.8 by adding NaOH.)
2. Open one end of the reactor and transfer the contents, as much as possible, into a labeled 500-mL Erlenmeyer flask.
3. To completely transfer the biomass, use 50 mL distilled water to wash the reactor. Pour this water and biomass mixture together into the flask.
4. Add 7.5 mL citrate buffer and 5 mL sodium azide solution into the flask to keep the pH constant and prevent the growth of microorganisms, respectively.
5. Add glacial acetic acid to each flask, using a titration tube, until the pH reaches 4.8. During acetic acid addition, continuously monitor the pH and stir using a magnetic bar. Record the volume of acetic acid added. This step should be done very carefully to prevent the pH from dropping below 4.8.
6. Add more water to bring the total liquid volume to 150.0 mL.
7. Place the flask inside the 100-rpm shaking air bath at 50°C.
8. When the temperature reaches 50°C, add 0.408 mL cellulase (filter paper activity ≅ 91 FPU/mL enzyme solution) and 0.852 mL cellobioase "Novozyme 188" (activity ≅ 250 CBU/g). This point on the time axis is considered as time zero (time = 0 h)
9. Withdraw 2.0 mL sample using a 5-mL pipet and a cut-off tip. To get a homogeneous sample, shake the flask slightly when withdrawing.
10. Place the flask back into the shaking air bath. Do not tightly cap the flask with a rubber stopper. (Note: This allows the air in the flask to expand as it warms.)
11. Boil samples for 15 min in screw-capped test tubes to denature the enzyme. Cool samples in cold water bath.
12. Close flasks with rubber stoppers after the flasks have been warmed for 10 min.
13. Using a 0.45-μm nylon membrane filter, filter samples into capped sample bottles. Store the samples in the freezer until the DNS assay, YSI glucose analysis, and/or HPLC analysis are ready to be performed. Ensure that each bottle is labeled clearly.
14. Repeat steps 9 to 13 as a function of time (i.e., 1, 3, 6, 10, 16, 24, 36, 48, and 72 h).
15. Perform DNS assay, YSI glucose analysis, and/or HPLC analysis to measure the concentrations of reducing sugars, glucose and/or xylose for each sample.

Enzymatic Hydrolysis Procedure for Acid Study

1. Place 140.0 mL of citrate buffer solution (0.05 M, pH 4.8) into twelve 500-mL Erlenmeyer flasks. The buffer solution has been prepared from a 1.0-M stock solution by diluting 20 fold. (Note: To prepare 1.0-M citrate buffer stock solution, see step 1 in "General Enzymatic Hydrolysis Procedure.")
2. Place various lime additions (0, 0.02, 0.05, 0.1, 0.2, and 0.3 g/g dry biomass) into each buffer-filled flask such that each lime addition is duplicated.
3. Add glacial acetic acid to each flask, using a titration burette, until the pH reaches 4.8. During acetic acid addition, continuously monitor the pH and stir using a magnetic bar. Record the volume of acetic acid added. This step should be done very carefully to prevent the pH from dropping below 4.8.
4. Add more water to bring the total liquid volume to 150 mL. Add 0.05 g of sodium azide (NaN_3) into each flask.
5. Add 7.5 g (105°C dry weight) of pretreated biomass to each flask.
6. Place the flasks inside the 100-rpm shaking air bath at 50°C.
7. When the temperature reaches 50°C, add 0.408 mL cellulase (filter paper activity ≅ 91 FPU/mL enzyme solution) and 0.852 mL cellobioase "Novozyme 188" (activity ≅ 250 CBU/mL). This point on the time axis is considered as time zero (time = 0.0).
8. Withdraw 2.0 mL sample using a 5-mL pipet and a cut-off tip. To get a homogeneous sample, shake the flask slightly when withdrawing.
9. Place the flasks back into the shaking air bath.
10. Boil samples for 15 min in screw-capped test tubes. Cool samples in cold water bath.
11. Close flasks with rubber stoppers after the flasks have been warmed for 10 min.
12. Using a 0.45-μm nylon membrane filter, filter samples into capped sample bottles. Store the samples in the freezer until the DNS assay and YSI glucose analysis are ready to be performed. Ensure that each bottle is labeled clearly.
13. Repeat steps 8 to 12 as a function of time (i.e., 1, 3, 6, 10, 16, 24, 36, 48, and 72 h).
15. Perform the DNS assay and YSI glucose analysis to measure reducing sugars and glucose concentration of each sample.
16. Repeat steps 1 to 15, but sulfuric acid will replace acetic acid to neutralize the lime.

Appendix D. Biomass Washing Procedure

Washing Procedure for Acid Study

1. Open one end of the reactors and transfer the contents (as much as possible) into six one-liter centrifuge bottles.
2. Wash the reactors with distilled water and place the rinse water into the centrifuge bottles.
3. Pour additional distilled water into the centrifuge bottles so that the water level reaches about 4/5 of total capacity.
4. Shake the centrifuge bottles to mix water and pretreated biomass thoroughly.
5. Put the centrifuge bottles in the centrifuge machine. Make sure that all six bottles are well balanced before the machine is turned on.
6. Centrifuge the bottles for ten minutes.
7. Carefully pour wash water in the bottles into the drain. Keep as much pretreated biomass remaining inside the bottles as possible.
8. Repeat steps 2 to 6 nine times.
9. Spread the completely washed biomass on aluminum foil. Air-dry it in the hood for at least two days.
10. After being completely air dried, the pretreated and washed biomass will be stored in Ziplock bags.
11. Determine the moisture content of the air-dried biomass as described in the NREL standard procedure No. 001.
12. Perform enzymatic hydrolysis as explained in Appendix C, "Enzymatic Hydrolysis Procedure for Acid Study."

Washing Procedure for Material Balances between Raw and Washed Only Biomass

1. Dry about 30 g of untreated biomass at 45°C for 24 hours or longer if necessary.
2. Place and cool the 45°C-dried biomass in the desiccator until it reaches room temperature.
3. Tare a 1-L centrifuge bottle. Transfer and weigh approximately 20 g of 45°C-dried biomass in the centrifuge bottle. Record the weight of the 45°C-dried biomass (W_1).
4. Using the rest of the 45°C-dried biomass, determine the moisture content as described in the NREL standard procedure No. 001 (X_1).
5. Place about 500 mL distilled water in the centrifuge bottle and stir for 15 minutes.
6. Centrifuge the water-biomass mixture at 3300 rpm for 5 minutes. Follow the instruction on the centrifuge machine.
7. During the centrifuge period, setup a vacuum filtration apparatus using a Buchner funnel and a 9-cm Whatman 934/AH glass fiber filter paper (particle retention = 1.5 μm). Weigh the 45°C-dried filter paper before setup. Record the value.
8. After centrifuging, carefully decant the water on the Buchner funnel with vacuum filtration. Decant as much water as possible. Observe the filtrate color.
9. Transfer as much filter cake into the centrifuge bottle as possible.
10. Repeat step 4 through 8 until the filtrate becomes clear. If it takes too long to filter, replace the old filter paper with a new one which has been dried and weighed in advance.
11. After being completely washed, transfer all the biomass in the centrifuge bottle, as well as the filter paper, into a container which has been weighed and recorded. Dry the biomass and filter paper at 45° C for 24 hours or longer if necessary.
12. Place and cool the biomass and filters in the desiccator until it reaches room temperature. Weigh them and record the values (W_2).

13. Using about 5 g of 45°C-dried washed biomass, determine the moisture content as described in the NREL standard procedure No. 001 (X_2). Store the rest of the biomass in the desiccator for determining the contents of ash, lignin, carbohydrate, and protein later.
14. The total weight loss due to washing is calculated using the following formula:

$$\text{Total Weight Loss} = \frac{W_1 \times (1 - X_1) - W_2 \times (1 - X_2)}{W_1 \times (1 - X_1)} \times 100\% \qquad (5)$$

where W_1 = 45°C-dried weight of raw biomass (g)
X_1 = moisture content of 45°C-dried raw biomass
W_2 = 45°C-dried weight of washed biomass (g)
X_2 = moisture content of 45°C-dried washed biomass

Washing Procedure for Material Balances between Raw and Pretreated & Washed Biomass

1. Dry about 30 g of untreated biomass at 45°C for 24 hours or longer if necessary.
2. Place and cool the 45°C-dried biomass in the desiccator until it reaches room temperature.
3. Weigh approximately 20 g of 45°C-dried biomass in a plastic weighing dish. Record the weight of the 45°C-dried biomass (W_1).
4. Using the rest of the 45°C-dried biomass, determine the moisture content as described in the NREL standard procedure No. 001 (X_1).
5. Under the optimal conditions, pretreat the biomass as explained in Appendix A.
6. Transfer as much pretreated biomass with 500 mL distilled water from the reactors to a centrifuge bottle and stir for 15 minutes.
7. Repeat steps 6 to 13 used in "Washing Procedure for Material Balances between Raw and Washed Only Biomass."
8. The total weight loss due to pretreatment and washing is calculated using the following formula:

$$\text{Total Weight Loss} = \frac{W_1 \times (1 - X_1) - W_2 \times (1 - X_2)}{W_1 \times (1 - X_1)} \times 100\% \qquad (6)$$

where W_1 = 45°C-dried weight of raw biomass (g)
X_1 = moisture content of 45°C-dried raw biomass
W_2 = 45°C-dried weight of pretreated and washed biomass (g)
X_2 = moisture content of 45°C-dried pretreated and washed biomass

Appendix E. Sugar Measurement

Dinitrosalicylic Acid (DNS) Assay

Reducing sugar was measured using the dinitrosalicylic acid (DNS) assay (Miller, 1959). A glucose standard prepared from the YSI 200 mg/dL glucose standard solution was used for the calibration, thus the reducing sugars were measured as "equivalent glucose." The step-by-step procedure is as follows:

DNS Reagent Preparation

1. Dissolve 10.6 g of 3,5-dinitrosalicylic acid crystals and 19.8 g NaOH in 1416 mL of distilled water.
2. Add 306 g Na-K-tartrate (Rochelle salts).
3. Melt phenol crystals under a fume hood at 50°C using a water bath. Add 7.6 mL of phenol to the above mixture.
4. Add 8.3 g sodium meta-bisulfite ($Na_2S_2O_4$).
5. Add NaOH, if required, to adjust the solution pH to 12.6.

DNS Reagent Calibration

1. Using a 200 mg/dL (i.e., 2 mg/mL) YSI glucose standard, prepare 1-mL samples in test tubes according to Table 12.
2. Place 0.5 mL of each sample into test tubes.
3. Dispense 1.5 mL of DNS reagent into each test tube using a 5-mL Brinckmann dispensette. (The following steps should be done under a fume hood to avoid exposure to phenol vapor.)
4. Place the caps on the tubes and vortex.
5. Boil samples in a water bath for 15 minutes.
6. Cool the test tubes for a few minutes. Add 8 mL of distilled water and vortex.
7. Zero the spectrophotometer (Milton Roy, Spectronic 1001) at 550 nm with distilled water (Note: to stabilize the spectrophotometer, it should be turned on for at least 1 h before using).
8. Measure the absorbance.
9. Prepare a calibration curve.

Reducing Sugar Measurement of Samples

1. Filter samples through a 0.45-μm nylon filter.
2. Accordingly, dilute the filtered samples into test tubes such that the sugar concentration lies between 0.2 to 1.0 mg/mL. Vortex the diluted samples.
3. Place 0.5 mL of each diluted sample into test tubes.
4. Repeat steps 3 to 8 used to prepare the calibration curve.
5. Calculate sugar concentration from the absorbance of the samples using the calibration curve.
6. Calculate the reducing sugar yield by following formula:

$$Y = S \times D \times V / W \qquad (7)$$

where
- Y = reducing sugar yield (mg equivalent glucose/ g dry biomass)
- S = sugar concentration in diluted sample (mg equivalent glucose/mL)
- D = dilution factor
- V = working liquid volume (mL)
- W = weight of dry biomass (g)

Table 12. Preparation of Glucose Standard Solutions for DNS Assay

Glucose Concentration (mg/mL)	200 mL/dL YSI Standard (mL)	Distilled Water (mL)
0.2	0.1	0.9
0.4	0.2	0.8
0.6	0.3	0.7
0.8	0.4	0.6
1.0	0.5	0.5

YSI Glucose Analysis

The YSI glucose analyzer offered a quick way to measure the glucose concentration of samples. The samples were directly injected into the analyzer and glucose concentration was read 30-60 seconds later. The YSI analyzer measures only D-glucose.

Preparation

1. Empty the waste container.
2. Check the log book to see if the buffer solution (YSI product number 2357) and membrane expired. Usually the buffer solution should be discarded after 5 days from when it was constituted and the membrane should be replaced 21 days after it was installed.
3. Prepare the buffer solution and/or replace the membrane if necessary. If the membrane is replaced, wait for at least 1 h for the block heater to reach a stable temperature.

Calibration

1. Press the clear button for a few seconds. Switch the YSI analyzer to run.
2. Press the clear button.
3. Zero the analyzer by adjusting the left nob and inject 25 μL of 200 mg/dL standard with the 25-μL syringe.
4. Wait until the display shows the measurement, then press the calibration button and adjust the display to read 200 mg/dL. Press the clear button.
5. Repeat steps 3 and 4 until the display reads 198-202 mg/dL when the 200 mg/dL standard is injected and -1 to 3 mg/dL when the clear button is pressed.
6. Inject 500 mg/dL standard to check the linearity. If the display reads 480-510 mg/dL, start injecting samples. If not, repeat this step again. If the reading still does not fall in the range, repeat steps 3 to 6 until the display reads 480-510 mg/dL when 500 mg/dL standard is injected.

Glucose Measurement of Samples

1. Filter the samples through 0.45-μm nylon filters.
2. Dilute the samples to 50-500 mg/dL.
3. Rinse the 25-μL syringe with the sample three times.
4. Inject the sample and wait for a reading.
5. Press the clear button after each measurement.
6. Calibrate the YSI analyzer with 200 mg/dL standard every 5 samples.

7. After finishing all the samples, switch the YSI analyzer to standby. Press the clear button for a few seconds to wash out the residual samples. Do not unplug the equipment unless it will not be used for more than 1 week.

HPLC Carbohydrate Analysis

Glucose, xylose, and cellobiose were measured using high performance liquid chromatography (HPLC). A Biorad Aminex HPX-87P column was used for carbohydrate separation. The instrumental conditions are as follows:

Sample injection volume:	20 μL
Eluant:	0.45-μm filtered reverse osmosis deionized (RODI) water
Flow rate:	0.6 mL/min
Column temperature:	85°C
Detector:	refractive index

The equipment used in HPLC are as follows:

Pump:	LDC Analytical Pump, constaMetric 3200
Autosampler:	Spectra-Physics, AS100
Column heater:	Jones Chromatography
RI detector:	LDC/Milton Roy, refractoMonitor III
Integrator:	Spectra-Physics, SP4270
RODI water:	NANOpure Ultrapure Water System

Carbohydrate Standard Preparartion

1. Prepare carbohydrate stock solutions: dissolve 0.5 g 45°C-dried carbohydrate (i.e., glucose, xylose, or cellobiose) in a 100-mL volumetric flask with distilled water.
2. Prepare 5 mL standard solutions in test tubes according to Table 13.
3. Place 0.6-1.0 mL standard solutions into autosampler sample vials.
4. Freeze the standard solutions if the analysis will be done later. (Note: Be sure to thoroughly mix the sample after thawing since freezing separates the sugars from the water.)

Equipment Setup

1. Filter about 4 L RODI water through a 0.45-μm nylon filter.
2. Connect HPLC equipment according to the Biorad manual, *Guidelines for Use and Care of Aminex Resin Based Columns*.
3. Turn on the pump, the autosampler, the RI detector, the water circulator, and the integrator. Also turn on the built-in refrigerator of the autosampler.
4. At a flow rate of 0.2 mL/min, turn on the column heater. Adjust the temperature setting to 85°C. Place a mercury thermometer in the column heater as an independent measurement. Usually it needs about 1 h to reach the desired temperature.
5. After the column heater reaches 85°C, gradually (i.e., increase 0.1 mL/min every minute) increase the flow rate to 0.6 mL/min. (Note: Do not operate the column at a flow rate greater than 0.2 mL/min at ambient temperature.)
6. Set the cycle time of the integrator to 20 minutes (for carbohydrate measurement) by pressing the dialog key and then manually entering the following information:

TIME	FUNCTION	VALUE
TT = 0.0	TF = PM	TV = 1
TT = 20.0	TF = ER	TV = 1

For other parameters, just use default values by pressing the enter key.

7. Adjust the energy nob on the detector to 5.
8. Press the edit button on the integrator to run a baseline. Adjust the zero nob on the detector to position the base line appropriately. If the baseline is straight and no noise is observed, start running the samples. If not, check if the temperature of the water circulator is constant.
9. Edit and load the autosampler file, as described in "Autosampler Setup."

Carbohydrate Measurement of the Samples

1. Filter the samples through 0.45-μm nylon filters.
2. Dilute the filtered samples such that the carbohydrate concentrations lie between 0.2 to 1.0 mg/mL.
3. Place 0.6-1.0 mL diluted samples into autosampler sample vials.
4. Place the samples and the standard solutions in the autosampler. Edit and load a sample file, as explained in "Autosampler Setup." Adjust the cycle time to 21 min for carbohydrate analysis.
5. Press the run button to start measurements.
6. Collect the chromatograms after all the samples are finished. Prepare a calibration curve according to the chromatograms of standard solutions. Calculate carbohydrate concentrations of the samples according to the calibration curve and the chromatograms of the samples.

Table 13. Preparation of Carbohydrate Standard Solutions for HPLC

Carbohydrate Concentration (mg/mL)	5 mg/mL Stock Solution (mL)	Distilled Water (mL)
0.2	0.2	4.8
0.4	0.4	4.6
0.6	0.6	4.4
0.8	0.8	4.2
1.0	1.0	4.0

Autosampler Setup

Editing/Loading Autosampler Files

1. Press the menu key to display the main menu. Sequentially select FILES, EDIT, and then INJECTION to display the edit menu using the arrow keys to move the cursor and the enter key to select.
2. Adjust the loop size to 20.0 μL and the number of injections per sample to 2 using the "+" or "-" key to increase or decrease the values, respectively. Adjust the cycle time to 21.0 minutes for carbohydrate analysis.
3. Turn on the built-in refrigerator by pressing the "+" key to switch the option from OFF to ON. Adjust the refrigerator temperature to 5°C using the "+" or "-" key.
4. Use the default values for other parameters in the autosampler file by continuously pressing the enter key.
5. Load the file by selecting FILES and LOAD from the main menu and then pressing the enter key.

Editing/Loading Sample Files

1. Press the sample key to display the sample menu.
2. Specify the sample set number.
3. Adjust the number of injections per sample and the cycle time as explained in "Editing/Loading Autosampler Files."
3. Specify the first sample vial to start with and the number of the samples using the "+" or "-" key.
4. Add the sample set to the queue by pressing the enter key.

Required Maintenance

1. Exactly follow the Biorad instruction to connect the equipment and operate the system. Any deviation from the instruction may destroy the resolution of the column.
2. Backwash the column overnight every time when the analysis is finished, according to the Biorad manual, *Guidelines for Use and Care of Aminex Resin Based Columns*. If there are many samples such that it would take more than three days to finish the analysis, backwash the column at least every 48 hours. The operating conditions for backwashing are as follows:

Eluant:	0.45-μm filtered RODI water
Flow rate:	0.1 mL/min
Column temperature:	ambient
Column direction:	reverse

3. Use fresh RODI water every time.
4. After the equipment is disconnected, cap the column and guard column with plastic end screws and store them in the refrigerator.

Troubleshooting

1. *Problem*: Unusual long peaks appear in the chromatogram.
   Cause: Air bubbles in eluant are detected by the RI detector.
   Solution:
   a. Replace the eluant with a fresh batch.
   b. Check if there is leaking along the tubes from the pump to the detector. If yes, reconnect the equipment or replace the leaking tube.
   c. Check if the tube connected to the outlet of the RI detector is capillary. If not, replace it with a capillary tube.

2. *Problem*: Carbohydrates do not separate well.
   Cause: The column is losing its resolution.
   Solution:
   a. Check the flow rate and column temperature.
   b. Check if the tubes connecting the autosampler and the guard column, the guard column and the column, and the column and the detector, are all capillary. If not, replace it with a capillary tube.
   c. Check if all the tubes are smooth enough. If any severe distortion is observed, replace that tube. (Note: The tubes should be as short and straight as possible, especially for the one connecting the column and the detector.)
   d. Check if there is leaking along the tube from the pump to the detector. If yes, reconnect the equipment or replace the leaking tube.
   e. Backwash the column overnight, as explained in "Required Maintenance."

f. Replace the guard column with a new one.

3. *Problem*: Baseline is out of the scale of the integrator.
   Cause: The temperature in the cell of the RI detector is not stable.
   Solution:
   a. Adjust the zero nob on the detector to position the baseline properly.
   b. Check if the temperature of the water circulator is higher than the ambient temperature. If not, adjust the temperature nob on the water circulator.
   c. Check the water level in the chamber of the water circulator. Add water if necessary.

Appendix F. Calcium Analysis

Calcium concentration was measured by the atomic absorption apparatus available in the Kinetics Group of the Chemical Engineering Department of Texas A&M University.

Preparation of Standard Solutions

1. Prepare 20 mg/mL KCl solution: place 10 g KCl in a 500-mL volumetric flask and then add RODI water to a total vomue of 500 mL.
2. Using 1000 ppm calcium reference solution (Fisher Scientific, Lot No. 940982-24), prepare blank and calcium standard solutions in 100-mL volumetric flasks according to Table 14.
3. Transfer the blank and calcium standard solutions to 50-mL disposable polypropylene centrifuge tubes.

Measurement of Samples

1. Filter samples through 0.22-μm nylon filters.
2. Dilute the filtered samples with 10 mL KCl solution and required amount of RODI water in 200-mL volumetric flasks such that the calcium concentrations lie between 1 to 4 ppm.
3. Transfer the diluted samples to 15-mL disposable polypropylene centrifuge tubes.
4. Zero the atomic absorption apparatus with the blank solution.
5. Measure the absorbances of calcium standard solutions. Plot a calibration curve.
6. Measure the absorbances of the samples. Calculate the calcium concentrations of samples using the calibration.

Table 14. Preparation of Blank and Calcium Standard Solutions for Atomic Absorption

Calcium Concentration (ppm)	1000 ppm Calcium Reference Solution (mL)	20 mg/mL KCl Solution (mL)	RODI Water (mL)
Blank	0.0	5.0	95.0
1.0	0.1	5.0	94.9
2.0	0.2	5.0	94.8
3.0	0.3	5.0	94.7
4.0	0.4	5.0	94.6

Appendix G. Ethanol Analysis

The ethanol concentrations were measured using gas chromatography (GC). The ethanol concentration was determined by mixing 0.1 mL of each filtered sample with 0.9 mL of 1 g/L methanol solution in RODI water and injecting it into the GC. By comparing the ratios of the peak areas of ethanol and methanol for standards, a calibration curve was determined and used to calculate the ethanol concentration for other samples. The equipment used for GC are as follows:

GC:	Hewlett Packard 5890A
Autosampler:	Hewlett Packard 7673A
Integrator:	Hewlett Packard 3396A

Preparation of Standard Solutions

1. Using a 250-mL volumetric flask, prepare 250 mL of 1-g/L methanol solution in RODI water: tare a 250-mL volumetric flask on an analytical balance, add 0.25 g pure methanol in the flask, then add RODI water until the total volume reaches 250 mL.
2. Using pure ethanol (200 proof), prepare standard solutions, just as the methanol solutions were prepared, in 100-mL volumetric flasks according to Table 15.
3. Mix 0.1 mL of each ethanol standard solution with 0.9 mL methanol solution in a GC vial. Vortex.

Measurement of Samples

1. Filter samples through 0.45-μm nylon filters.
2. If necessary, dilute the filtered samples with RODI water such that the ethanol concentrations lie between 5 and 50 g/L.
3. Mix 0.1 mL of each diluted sample with 0.9 mL methanol solution in a GC vial. Vortex.
4. Arrange the standard vials and the sample vials on the tray for the autosampler. Press the start button to start the measurements. Note that the GC requires about 1 h to warm up.
5. Plot a calibration curve. Use the calibration curve to calculate the ethanol concentration of the samples.

Table 15. Preparation of Ethanol Standard Solutions

Ethanol Concentration (g/L)	Pure Ethanol (g)[a]
5	0.5
10	1.0
20	2.0
30	3.0
50	5.0

a. Based on a total volume of 100 mL.

Appendix H. Experimental Data

Acid Study

Table 16. Acid Study Data (Neutralized by Acetic Acid)[a,b]

Time (h)	Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Lime Additions					
	0 g lime/g dry biomass	0.02 g lime/g dry biomass	0.05 g lime/g dry biomass	0.1 g lime/g dry biomass	0.2 g lime/g dry biomass	0.3 g lime/g dry biomass
1	116.64	125.44	120.64	118.36	108.80	104.32
3	256.32	261.28	254.56	252.16	247.52	235.52
6	288.24	302.99	302.86	287.29	282.45	275.90
10	341.08	345.35	338.85	341.96	320.26	328.74
18	373.89	387.72	372.57	364.28	354.88	354.71
24	424.04	418.85	413.77	412.21	380.09	372.92
36	434.51	443.51	447.03	427.35	404.37	387.18
48	432.16	455.27	427.75	425.46	410.49	401.76
72	465.07	490.53	463.96	458.38	442.46	419.16

a. Data for Fig. 1a and 2.

b. Pretreatment conditions: 100°C, 1 h, 0.1 g lime/g dry biomass, 10 mL water/g dry biomass, 40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

Table 17. Acid Study Data (Neutralized by Sulfuric Acid)[a,b]

Time (h)	Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Lime Additions					
	0 g lime/g dry biomass	0.02 g lime/g dry biomass	0.05 g lime/g dry biomass	0.1 g lime/g dry biomass	0.2 g lime/g dry biomass	0.3 g lime/g dry biomass
1	134.64	143.05	83.70	14.28	-0.30	0.10
3	232.07	239.13	172.11	20.20	22.60	23.40
6	296.53	314.41	187.44	32.37	24.85	24.60
10	296.53	350.92	228.91	28.31	23.50	30.91
18	455.32	421.32	303.37	11.84	-1.81	-0.60
24	460.52	444.69	274.28	12.88	0.27	-0.94
36	467.79	471.70	292.98	-2.71	-1.81	-0.25
48	398.70	338.72	246.23	-9.98	-0.77	0.44
72	413.76	444.69	267.52	-9.98	-1.29	-0.94

a. Data for Fig. 1b and 2.

b. Pretreatment conditions: 100°C, 1 h, 0.1 g lime/g dry biomass, 10 mL water/g dry biomass, 40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

Optimization of Pretreatment Condictions

Table 18. Optimization of Pretreatment Time and Temperature[a,b,c]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Pretreatment Times					
	1 h	3 h	6 h	10 h	16 h	24 h
60	369.95	489.14	500.97	469.70	526.33	511.96
80	400.00	501.82	513.65	523.79	549.15	544.08
90	436.73	486.60	523.79	526.33	516.19	508.58
100	503.51	530.56	529.71	523.79	550.00	495.05
110	460.40	478.15	539.01	523.79	555.92	499.28
120	524.64	532.25	540.70	566.90	573.67	541.55
130	527.18	535.63	533.09	549.15	555.07	498.44

a. Data for Fig. 3a and 4.

b. Pretreatment conditions: 0.1 g lime/g dry biomass, 10 mL water/g dry biomass, 40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. Experiments performed by Dr. Barry Burr.

Table 19. Optimization of Pretreatment Time[a,b,c]

Pretreatment Time (h)	3-d Corrected Reducing Sugar Yield (mg eq. glucose/g dry biomass)
1.0	447.18[d]
1.5	468.90[d]
2.0	483.07[d]
3.0	485.90[d]
3.0	481.18[e]

a. Data for Fig. 3b.

b. Pretreatment conditions: 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. Experiments performed by Dr. Barry Burr.

d. Boil-up.

e. Heat-up.

Table 20. Optimization of Lime Loading[a,b,c]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Lime Loadings					
	0.01 g lime/g dry biomass	0.05 g lime/g dry biomass	0.1 g lime/g dry biomass	0.15 g lime/g dry biomass	0.2 g lime/g dry biomass	0.3 g lime/g dry biomass
100	126.67	202.78	529.43	574.11	572.00	571.55
120	159.20	257.96	523.70	564.43	524.61	559.45

a. Data for Fig. 5.

b. Pretreatment conditions: 3 h, 10 mL water/g dry biomass, 40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. Experiments performed by Dr. Barry Burr.

Table 21. Optimization of Water Loading[a,b,c]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Water Loadings					
	5 mL water/g dry biomass	7 mL water/g dry biomass	9 mL water/g dry biomass	11 mL water/g dry biomass	13 mL water/g dry biomass	15 mL water/g dry biomass
100	237.51	480.79	504.18	498.73	499.51	503.41
120	274.15	486.25	515.88	522.90	574.36	543.95

a. Data for Fig. 6.

b. Pretreatment conditions: 3 h, 0.1 g lime/g dry biomass, 40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. Experiments performed by Dr. Barry Burr.

Table 22. Partical Size Study[a,b,c]

Partical Size (mesh)	3-d Corrected Reducing Sugar Yield (mg eq. glucose/g dry biomass)
4 to 10	382.01
10 to 20	437.73
20 to 40	512.34
40 to 80	501.01
80 and finer	508.57
a. Data for Fig. 7.	
b. Pretreatment conditions: 0.1 g lime/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.	
c. Experiments performed by Dr. Barry Burr.	

Enzyme Loading Studies

Table 23. Enzyme Loading Studies — Run 1 (for Pretreated Switchgrass)[a,b,c]

Cellulase Loading (FPU/g dry biomass)	3-d Glucose Yield[d] (mg/g dry biomass)	3-d Reducing Sugar Yield (mg eq.glucose/g dry biomass)
0.5	170.5	400.0
1.0	193.9	440.6
2.5	223.3	506.7
5.0	251.7	553.9
10.0	281.2	596.4
30.0	303.5	630.4
a. Data for Fig. 9a and 9c.		
b. Pretreatment conditions: 120°C, 3 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass.		
c. Experiments performed by Dr. Barry Burr.		
d. Measured using YSI.		

Table 24. Enzyme Loading Studies — Run 2 (for Pretreated Switchgrass)[a,b]

Cellulase Loading (FPU/g dry biomass)	3-d Glucose Yield[c]		3-d Xylose Yield[c]		3-d Total Sugar Yield	
	(mg/g dry biomass)	(%)	(mg/g dry biomass)	(%)	(mg/g dry biomass)	(%)
0	6.41	1.52	106.11	41.68	112.52	16.66
1	152.29	36.21	179.08	70.34	331.37	49.08
3	202.63	52.70	175.01	68.75	396.67	58.75
5	243.06	63.06	179.41	70.48	444.67	65.86
10	279.32	71.83	198.39	77.93	500.52	74.13
25	308.98	73.46	223.54	87.81	532.51	78.87
50	323.97	77.02	229.13	90.00	553.10	81.92
75	321.17	76.36	234.56	92.14	555.73	82.31
100	330.80	78.65	247.41	97.18	578.21	85.64

a. Data for Fig. 9.

b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass.

c. Measured using HPLC.

Table 25. Enzyme Loading Studies — Run 2 (for Untreated Switchgrass)[a,b]

Cellulase Loading (FPU/g dry biomass)	3-d Glucose Yield[c]		3-d Xylose Yield[c]		3-d Total Sugar Yield	
	(mg/g dry biomass)	(%)	(mg/g dry biomass)	(%)	(mg/g dry biomass)	(%)
0	28.16	6.69	1.93	0.76	30.08	4.46
5	84.90	20.19	10.95	4.30	95.85	14.20
25	101.57	24.15	19.04	7.48	120.61	17.86
50	103.90	24.70	20.37	8.00	124.27	18.40
100	111.15	26.43	22.64	8.89	133.79	19.81

a. Data for Fig. 9 and 10.

b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass.

c. Measured using HPLC.

Table 26. Reducing Sugars Resulting from Enzymes[a,b]

Enzyme	Enzyme Loading (FPU/g dry biomass or CBU/g dry biomass)	3-d Reducing Sugar Yield (mg eq. glucose/g dry biomass)
Cellulase (FPU)	10.0	1.6
	20.0	3.8
Cellobiase (CBU)	14.2	1.5
	28.4	3.7
	56.8	8.2
	113.6	17.1
a. Data for Fig. 10a.		
b. Experiment performed by Dr. Barry Burr.		

Table 27. Glucose Resulting from Enzymes[a]

Cellulase Loading (FPU/g dry biomass)	Cellobiase Loading (CBU/g dry biomass)	3-d Glucose Yield[b] (mg/g dry biomass)
0	28.4	2.48
50	28.4	4.07
100	28.4	5.66
a. Data for Fig. 10b.		
b. Measured using HPLC.		

Enzymatic Hydrolysis Profiles

Table 28. Hydrolysis Profiles [a,b]

Time (h)	Glucose Yield (mg/g dry biomass)	Xylose Yield (mg/g dry biomass)	Cellobiose Yield (mg/g dry biomass)	Total Sugar Yield (mg/g dry biomass)	Corrected Reducing Sugar Yield (mg eq. glucose/g dry biomass)
0	9	0	11	20	45
1	53	14	36	104	176
3	105	31	4	140	330
6	134	54	0	188	378
10	152	75	0	227	421
16	175	127	0	302	467
24	204	120	0	324	489
36	219	120	0	339	501
48	233	158	0	391	521
72	244	148	0	392	538

a. Data for Fig. 11 and 12.

b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

Lime Recovery

Table 29. Optimization of pH for Lime Recovery[a,b]

pH	Calcium Concentration (mg/mL)
6.5	2.628
7.0	2.448
7.3	2.226
7.7	2.216
8.3	2.346
9.0	2.340
9.9	2.490
a. Data for Fig. 13a.	
b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh.	

Table 30. Lime Recovery[a,b]

Wash Times	Calcium Concentration (mg/mL)	
	Before Carbonation	After Carbonation
1	2.529	2.490
2	1.225	1.221
3	0.600	0.568
4	0.353	0.316
5	0.258	0.248
6	0.199	0.176
7	0.292	0.167
8	0.176	0.146
9	0.147	0.136
10	0.145	0.105
a. Data for Fig. 13b.		
b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh.		

Simultaneous Saccharafication/Fermentation

Table 31. Initial Hydrolysis Rate Study under SSF[a,b]

Time	Sugar Concentration (mg/mL)			Sugar Yield (%)	
(h)	Glucose	Xylose	Cellobiose	Glucose	Xylose
0	0.18	0.00	0.01	1.79	0.00
1	0.51	0.00	0.52	9.54	0.00
3	1.47	0.17	1.01	23.00	2.58
6	2.08	0.54	1.00	28.49	8.34
12	2.81	1.18	0.93	34.46	18.14
24	3.58	2.36	0.32	35.53	36.20
a. Data for Fig. 15.					
b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh. Hydrolysis conditions: 25 FPU cellulase/g cellulose.					

Table 32. Sugar Concentrations during SSF[a,b,c]

Time	Pretreated Switchgrass			Cellulose Standard		
(d)	Glucose Concentration[d] (mg/mL)		Cellobiose Concentration	Glucose Concentration[d] (mg/mL)		Cellobiose Concentration
	Run 1	Run 2	(mg/mL)	Run 1	Run 2	(mg/mL)
1	0.69	0.97	1.40	0.14	0.18	0.43
2	0.43	1.28	0.53	0.07	0.08	0.19
3	-----	1.86	0.45	-----	0.11	0.13
4	0.50	2.33	0.17	0.10	0.09	0.02
5	0.63	2.87	0.21	0.11	0.07	0.01
6	0.78	3.09	0.16	0.14	0.09	0.01
7	1.04	3.43	0.32	0.06	0.09	0.01
a. Data for Fig. 16a.						
b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh.						
c. Experiment Run 1 was performed by Dr. Barry Burr.						
d. Glucose concentrations were measured using YSI in Run 1 and using HPLC in Run 2.						

Table 33. Ethanol Yields during SSF[a,b,c]

Time	Pretreated Switchgrass		Cellulose Standard	
(d)	Run 1	Run 2	Run 1	Run 2
0	0%	0%	0%	0%
1	39%	44%	23%	42%
2	60%	48%	30%	58%
3	-----	48%	-----	71%
4	64%	48%	40%	80%
5	68%	49%	51%	86%
6	71%	47%	76%	87%
7	72%	47%	74%	91%
a. Dta for Fig. 16b.				
b. Pretreatment conditions: 120°C, 2 h, 0.1 g lime/g dry biomass, 9 mL water/g dry biomass, 40 mesh.				
c. Experiment Run 1 was performed by Dr. Barry Burr.				

Appendix I. Calculations

Residual calcium in the pretreated biomass:

$$0.025\ \frac{\text{g calcium}}{\text{g biomass}} \times \frac{\text{mol Ca}}{40.08\ \text{g calcium}} = 6.237 \times 10^{-4}\ \text{mol Ca / g biomass} \tag{8}$$

The amounts of the acid groups from the organic acids produced in alcoholic fermentations:

$$0.0023\ \frac{\text{g acetic acid}}{\text{g sugar}} \times \frac{\text{mol acetic acid}}{60.05\ \text{g acetic acid}} \times \frac{1\ \text{mole acid group}}{1\ \text{mol acetic acid}} = 3.83 \times 10^{-5}\ \frac{\text{mol acid groups}}{\text{g sugar}} \tag{9}$$

$$0.0016\ \frac{\text{g butyric acid}}{\text{g sugar}} \times \frac{\text{mol butyric acid}}{88.10\ \text{g butyric acid}} \times \frac{1\ \text{mole acid group}}{1\ \text{mol butyric acid}} = 1.82 \times 10^{-5}\ \frac{\text{mol acid groups}}{\text{g sugar}} \tag{10}$$

$$0.0017\ \frac{\text{g formic acid}}{\text{g sugar}} \times \frac{\text{mol formic acid}}{46.02\ \text{g formic acid}} \times \frac{1\ \text{mole acid group}}{1\ \text{mol formic acid}} = 2.325 \times 10^{-5}\ \frac{\text{mol acid groups}}{\text{g sugar}} \tag{11}$$

$$0.00190\ \frac{\text{g lactic acid}}{\text{g sugar}} \times \frac{\text{mol lactic acid}}{90.08\ \text{g lactic acid}} \times \frac{1\ \text{mole acid group}}{1\ \text{mol lactic acid}} = 2.109 \times 10^{-5}\ \frac{\text{mol acid groups}}{\text{g sugar}} \tag{12}$$

$$0.00177\ \frac{\text{g succinic acid}}{\text{g sugar}} \times \frac{\text{mol succinic acid}}{118.09\ \text{g succinic acid}} \times \frac{2\ \text{mole acid group}}{1\ \text{mol succinic acid}} = 2.998 \times 10^{-5}\ \frac{\text{mol acid groups}}{\text{g sugar}} \tag{13}$$

$$\text{Total amount of the acid groups} = 1.3 \times 10^{-4}\ \frac{\text{mol acid groups}}{\text{g sugar}} \tag{14}$$

The calcium consumed by the acid groups:

$$1.3 \times 10^{-4}\ \frac{\text{mol acid groups}}{\text{g sugar}} \times \frac{0.6786\ \text{g sugar}}{\text{g biomass}} \times \frac{1\ \text{mole Ca}}{2\ \text{mol acid groups}} = 4.44 \times 10^{-5}\ \frac{\text{mol Ca needed}}{\text{g biomass}} \tag{15}$$

ABSTRACT

Lime (calcium hydroxide) was used as the pretreatment agent to enhance the enzymatic digestibility of switchgrass. The effect of lime pretreatment on digestibility at various pretreatment conditions has been studied. The optimal pretreatment conditions were: time = 2 h, temperature = 100°C and 120°C, lime loading = 0.1 g/g dry biomass, water loading = 9 mL/g dry biomass. Studies on the effect of particle size indicate that there was little benefit of grinding below 20-40 mesh; even coarse particles (4-10 mesh) digested well. The effect of cellulase loading for enzymatic hydrolysis has been studied and effective hydrolysis was obtained with 5 FPU/g dry biomass. High sugar yields (79% for glucose and 97% for xylose) were obtained due to the lime pretreatment. Under the optimal pretreatment and hydrolysis conditions, the glucose yield was 3 times that of untreated switchgrass, the xylose yield was 11 times, and the total sugar yield was 4.3 times. Using simultaneous saccharification/fermentation, the best ethanol yield was 70% of the theoretical yield. A material balance study showed that little glucan (ca. 10%) was solublized due to the lime pretreatment whereas about 26% of xylan and 29% of lignin became solublized. The lime could not be recovered by carbonating the wash water because the calcium was complexed with soluble organics. To overcome this limitation, three alternative lime recovery processes are presented.

FINAL REPORT

DEVELOPMENT OF ALTERNATIVE PRETREATMENT AND BIOMASS FRACTIONATION PROCESSES: LIME PRETREATMENT

NREL SUBCONTRACTOR REPORT

Part II
Economic Analysis

by

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Under Prime Contract DE-AC02-83CH10093

July 1996

Summary

In this report, a preliminary economic analysis of the lime pretreatment process is presented. A number of cases are described according to the following table:

Process A Lime treated biomass is washed with water which is subsequently carbonated to recover the calcium as calcium carbonate.						Estimated Cost ($/tonne dry biomass)
	Lime kiln	Lime kiln fuel	Make-up lime	Lime recovery	Saccharification/ fermentation pH	
Process A-1	yes	natural gas	purchased lime	70%	2-11	17.55
Process A-2	yes	natural gas	purchased limestone	70%	2-11	18.53
Process A-3	yes	lignin	purchased lime	70%	2-11	16.60
Process A-4	no	-----	purchased lime	0%	2-11	19.92
Process B Lime-treated biomass is washed with water which is sent to a biodigester which converts solubilized organics to methane and carbon dioxide. The carbon dioxide reacts with the calcium to form calcium carbonate.						Estimated Cost ($/tonne dry biomass)
	Lime kiln	Lime kiln fuel	Make-up lime	Lime recovery	Saccharification/ fermentation pH	
Process B	yes	biogas	purchased lime	85%	2-11	10.58
Process C Lime-treated biomass is carbonated directly in place. The resulting calcium bicarbonate is recovered with the fermentor solids.						Estimated Cost ($/tonne dry biomass)
	Lime kiln	Lime kiln fuel	Make-up lime	Lime recovery	Saccharification/ fermentation pH	
Process C	yes	lignin	purchased lime	95%	>5.5	7.65

The cost of the pretreatment ranges from $7.65-$19.32/tonne depending which process option is selected. These costs are attractive compared to many other pretreatment processes.

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Process Description

The lime may be recovered from the pretreatment process by washing the biomass with water and carbonating the wash in order to precipitate $CaCO_3$ (Process A, see Figure 1). If the calcium is complexed with organic matter, it will not precipitate by carbonation. Instead, the organic matter must be biologically digested to methane and carbon dioxide. In this case, the carbon dioxide reacts with the lime which precipitates as $CaCO_3$ (Process B, see Figure 2). In a third option, the lime-treated biomass is contacted directly with carbon dioxide to neutralize excess lime (Process C, see Figure 3). Processes A and B attempt to completely remove calcium from the pretreated biomass which gives more flexibility in the choice of saccharification/fermentation pH. Process C is restricted to a saccharification/fermentation pH greater than 5.5, a pH attainable using CO_2 as the acid. Lower pH requires a stronger acid (e.g., sulfuric acid) which would react with the calcium to form gypsum, an undesirable waste product.

The equipment for the three processes is listed in Tables 1, 2, and 3. The design is based on a raw biomass feed rate of 40 tonne/h. The design calculations and costs for the process equipment are fully described for Process A. For Process B, calculations for the biodigester and the lime kiln are also shown since they differ markedly from Process A. The equipment for Process C is a subset of the equipment for Process A, so additional design calculations are not needed for this process.

Grinding

The raw biomass is assumed to be dry or semi-dry. It is ground to a -40 mesh particle size in a "burr mill" (G1). A burr mill is an attrition mill much like the traditional stone mills used to grind grain into flour. It has two circular plates, one is stationary and the other rotates. The surfaces of these plates are coated with "Ni-hard," a very hard alloy of nickel and cast iron. The Ni-hard has a waffle pattern which grinds the biomass. The biomass is fed at the center of the rotating disc, and as it travels toward the disc periphery, it is ground by the waffle patterns. Adjusting the clearance between the rotary and stationary plates determines the particle size. By setting them close together, it is possible to obtain -200 mesh particles, but this is not necessary for this process. The plates do wear and must be replaced every 6-8 months at a cost of $400 per machine. An extra burr mill is kept in reserve which allows the process to continue operating during maintenance. In this process, two burr mills operate while one is kept in reserve. Each one costs $23,400 with attatchments, and is capable of grinding 25 tons of biomass per hour. About 120 hp are required to operate the machines which results in 2.45 kWh of electricity being used per tonne of grass.

Pretreatment

After being ground, the biomass is transferred to the pretreatment tank (V1) using a screw conveyor (A1). Live steam, pre-heated water, and lime are added to the tank and mixed with the biomass. The slurry is held at 120°C for 2 hours (100°C in Process B). The pretreatment vessel is a 870 m^3 cylindrical concrete tank which costs about $85,000. The slurry is mixed using a 3-hp stainless steel, dual impeller agitator (M1). It costs about $10,500 and uses 0.056 kWh per tonne of switchgrass. The reason that Process B uses 100°C pretreatment rather than 120°C pretreatment is that the higher temperature makes more of the biomass (mainly xylan and glucan) water soluble. All water soluble biomass is sent to the kiln in Process B and is used for boiler fuel rather than fermentation stock. The lower pretreatment temperature only slightly reduces digestibility, if at all.

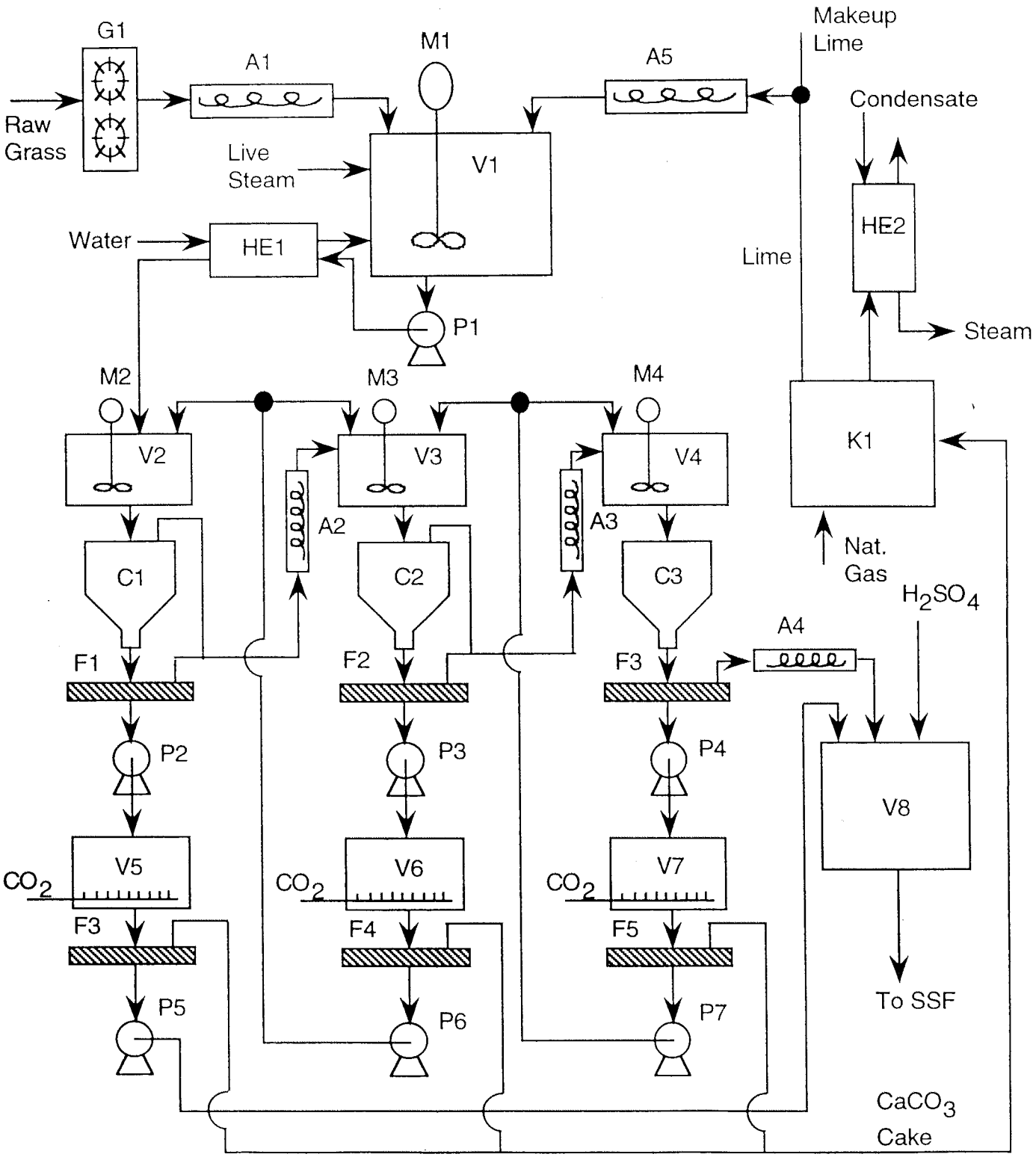

Figure 1. Lime pretreatment process (Process A)

Table 1. Equipment Legend for Process A

Item Label	*Description*
G1	Grinder (Burr Mill)
A1	Grass Feed Conveyor
V1	Pretreatment Tank
M1	Pretreatment Mixer
P1	Pretreated Slurry Pump
HE1	Feed Pre-Heat Exchanger
V2,V3,V4	Wash Vessels
M2,M3,M4	Wash Vessel Mixers
V5,V6,V7	Limestone Precipitation Vessels
C1,C2,C3	Hydrocyclones
F1,F2,F3	Polishing Filters
F4,F5,F6	Limestone Filters
P2,P3,P4,P5,P6,P7	Wash and Precipitation Transfer Pumps
A2,A3,A4	Washed Grass Conveyors
V8	Product Mixing Vessel
A5	Lime Conveyor
K1	Lime Kiln
HE2	Kiln Heat Exchanger

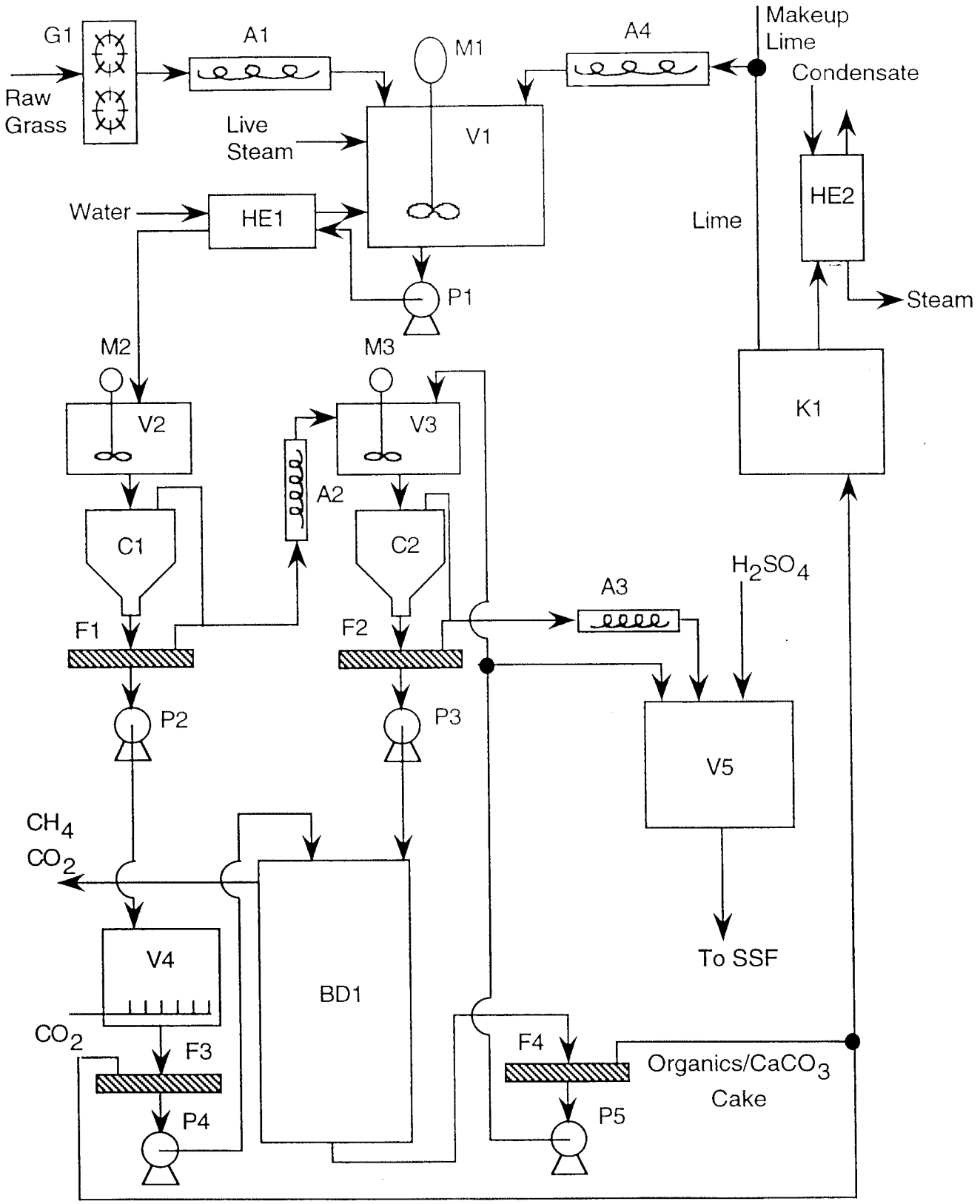

Figure 2. Lime pretreatment process with bio-digester (Process B)

Table 2. Equipment Legend for Process B

Item Label	*Description*
G1	Grinder (Burr Mill)
A1	Grass Feed Conveyor
V1	Pretreatment Tank
M1	Pretreatment Mixer
P1	Pretreated Slurry Pump
HE1	Feed Pre-Heat Exchanger
V2,V3	Wash Vessels
M2,M3	Wash Vessel Mixers
V4	Limestone Precipitation Vessel
C1,C2	Hydrocyclones
F1,F2	Polishing Filters
F3	Limestone/Slough/Lignin Filter
P2,P3,P4	Wash and Precipitation Transfer Pumps
A2,A3	Washed Grass Conveyors
V5	Product Mixing Vessel
A4	Lime Conveyor
BD1	Fixed-Film Anaerobic Bio-Digester
K1	Lime Kiln
HE2	Kiln Heat Exchanger

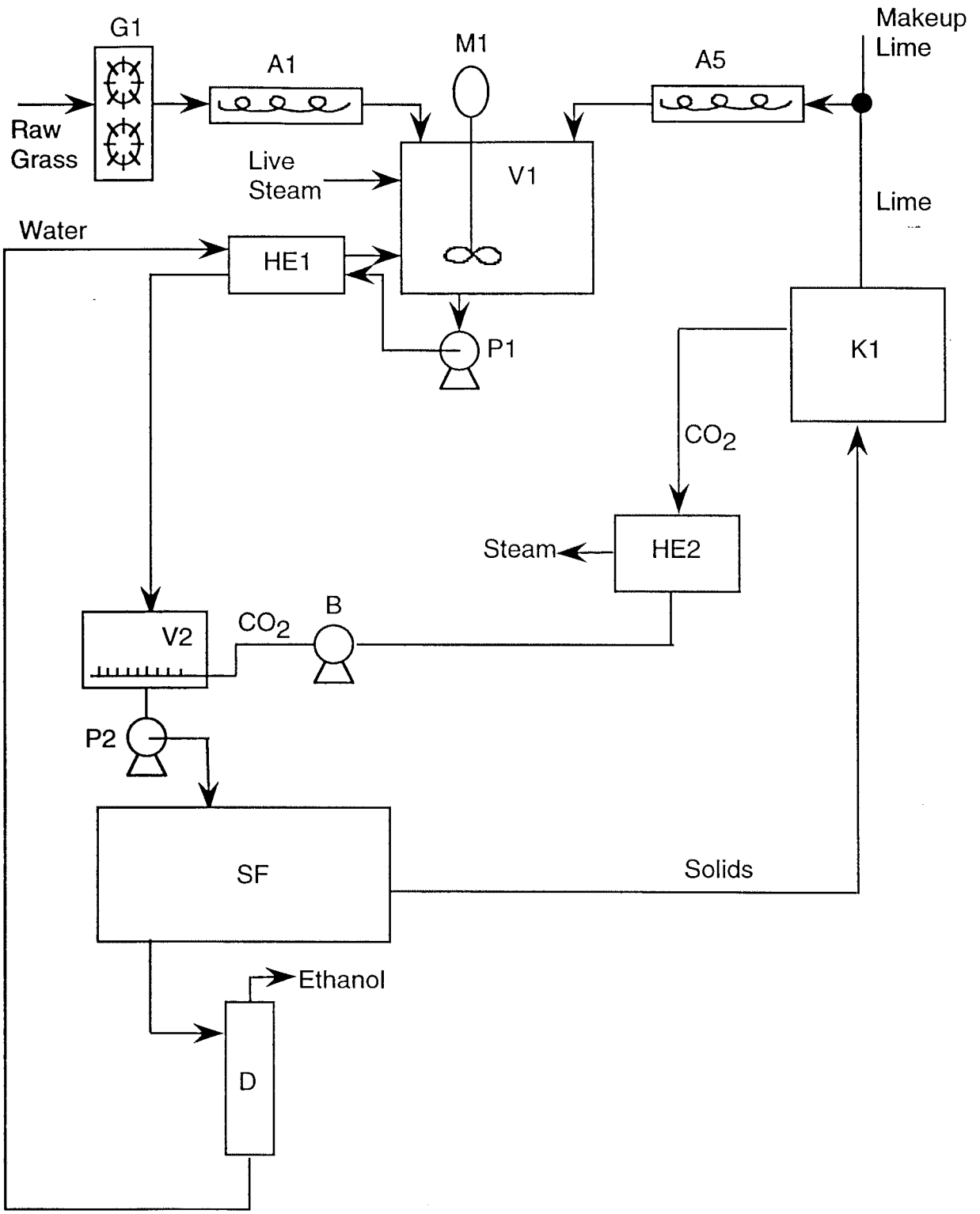

Figure 3. Lime pretreatment process (Process C)

Table 3. Equipment Legend for Process C

Item Label	*Description*
G1	Grinder (Burr Mill)
A1	Grass Feed Conveyor
A2	Lime feed Conveyor
V1	Pretreatment Tank
M1	Pretreatment Mixer
P1,P2	Slurry Pumps
HE1	Feed Pre-Heat Exchanger
V2	Carbonation Vessel
B	CO_2 Blower
SF	Saccharification/Fermentation Vessels
D	Distillation
K1	Lime Kiln
HE2	Kiln Heat Exchanger

Slurry Cooling

The hot, pretreated slurry is pumped (P1) through a heat exchanger (HE1) where the slurry is cooled to 38°C by heating pretreatment water for the next batch to 112°C. Extra heat required to raise the water, biomass, and lime temperatures to 120°C is supplied by adding steam directly to the pretreatment vessel. The slurry pump sends 1660 gpm throught the heat exchanger. This pump costs about $3,500 and uses 0.61 kWh per tonne biomass. The heat exchanger is a 16 foot long steel heat exchanger having 1-1/4" tubes which gives it a heat transfer surface area of 6,300 ft^2. This heat exchanger costs about $38,000. Also, 0.075 tonne steam per tonne grass are required to heat the slurry to the final pretreatment temperature.

Slurry Dewatering

After pretreatment, the calcium in the slurry must be recovered in the form of $CaCO_3$ so that it can be reacted to CaO in the lime kiln. This is accomplished by filtering the bio-solids from the slurry, and contacting the filtrate with CO_2 to precipitate $CaCO_3$. This step is repeated three times in a counter-current batch system shown in Figure 1. In Process A, it is assumed that 70% of the calcium can be recovered using this technique. This has been the case with bagasse (Nagwani, 1992). However, it was not possible to do this with switchgrass. This design is included, though, because it is the most likely process to be used to pretreat biomass.

Dewatering the slurry in each batch is accomplished by mixing the biomass with the proper amount of post-precipitation water from a downstream batch and then recovering the water using a hydrocyclone and a polishing filter. The wash vessels (V2, V3, and V4) handle 623 tonnes of slurry per hour and have a residence time of 0.5 hour, so the tank working volume is about 360 m^3. The concrete vessels cost $47,000 each. The mixers (M2, M3, and M4) for each vessel are 2-hp agitators which cost $10,000 each and consume 0.07 kWh per tonne grass. Hydrocyclones (C1, C2, and C3), which remove most of the water, are made of steel and are estimated to cost $55,000 each. The polishing filters remove enough extra water so that the cake is 20% solids. For this task, a 10" bowl centrifugal filter with a 75-hp motor is acceptable. The filter with drive and motor cost $97,500 each.

Calcium Precipitation

The filtrate obtained from the previous step contains soluble organics and dissolved lime. The filtrate is pumped into a precipitation vessel (V5, V6, and V7) where lime is converted into limestone by bubbling CO_2 through the liquid. The limestone is insoluble and precipitates. The limestone is filtered from the liquid and sent to the lime kiln. The filtrate is sent to an upstream wash vessel. The filtrate from the first precipitation is sent to the final product mixing vessel.

The precipitation vessels are made of concrete, can hold 360 m^3, and cost $47,000 each. Attatched to each vessel is a CO_2 blower which injects 664 ft^3 CO_2 per minute. The blowers are 10-psi rotary blowers which cost $12,000 each. Blowing CO_2 into the contents requires 1.13 kWh per tonne grass. Filtering the precipitated limestone (20% solids in cake) can be accomplished using a 40-in bowl centrifugal filter (F3, F4, and F5) with a 75-hp motor and drive. Filter equipment costs about $98,000 each and energy requirements are a combined 8.4 kWh per tonne biomass for all filters. Pumps (P2 - P7) for transferring slurries and liquids in the wash and precipitation steps must handle 5,470 gpm and have 30 psi head. API 610 cast steel pumps of this type cost $16,000 each and cumulatively consume 8.93 kWh per tonne grass. Conveyors or augers (A2, A3, and A4) for transporting washed grass are assumed to be 30 ft long.

Estimated equipment cost for the conveyors is $11,000 each. Energy requirement is 0.17 kWh per tonne grass each.

Lime Kilning

For Process A, the limestone filter cakes from the precipitation steps are sent to a lime kiln where the limestone is converted into lime. Excess heat in the exhaust gases is used to make 100-psig steam. The steam can be used in some other part of the plant, so it has value which can be credited to the project.

A lime kiln is a horizontal rotary kiln with refractory brick. The calcium carbonate plus water and organics, is charged to one end while the entire kiln rotates. The solids tumble in the lime kiln and crack as hot combustion gases pass through the center of the kiln. Commercial lime kilns operate at about 900°C. The gaseous effluent will consist primarily of CO_2 released from the limestone and nitrogen from the combustion gases. This effluent is so hot that useful energy can be produced to make steam for electricity generation or process heat. A lime kiln sufficiently large to produce all the lime consumed by this process must be 8-ft in diameter by 140-ft long in Process A. It represents the single largest expense in the process. The kiln also requires 0.56 kWh per tonne biomass for drive energy and 22.9 MM Btu gas per hour for fuel.

Economic Analysis

The lime pretreatment plant design cases are all based on processing 40 dry tonnes of biomass per hour. The plant will operate 8000 h/yr and return on investment (ROI) will be set to 15%. Unless otherwise specified, the costs for purchased equipment were obtained from *Plant Design and Economics for Chemical Engineers* by Peters and Timmerhaus (1991). To account for costs of installation and piping, Lang factors are applied to purchased equipment costs; these also are obtained from Peters and Timmerhaus (1991). Marshall and Swift price indexes are applied to installed equipment costs to update costs to July 1994. The total of all adjusted equipment costs is the fixed capital investment (FCI). Several of the plant's operational expenses can be expressed as functions of FCI. These are depreciation (10% FCI), local tax (3% FCI), insurance (0.7% FCI), maintenance (4% FCI), and interest (1.15 × 15% FCI). These annualized expenses are added to the annual labor, utilities, and raw materials costs to give a total annual cost. This cost is divide by the amount of biomass processed annually to give a per-tonne lime pretreatment cost. The biomass is assumed to be free, so that the cost will represent processing only. Unit costs for utilities and raw materials are shown in Table 4.

Assumptions

In order to make a cursory design of a lime pretreatment plant, it was necessary to make several process assumptions. These were based often on educated guesses and can only be refined with experimental studies for which resources are not currently available. These assumptions are listed in Table 5.

Table 4. Utilities and Raw Materials Costs

Commodity	Unit	Unit Price ($/unit)
Switchgrass	tonne	0
Lime	tonne	57.2
Limestone	tonne	15
Sulfuric Acid	tonne	75
Steam (100 psig)	tonne	4.89
Electricity	kWh	0.02
Natural Gas	MM Btu	1.75

Table 5. Process Assumptions

Ethanol yield = 100 gal/tonne pretreated biomass
Biomass moisture = 6 wt% water
Wash and precipitation cakes = 80 wt% water
Cakes to be kilned = 50% water
Calcium recovery (Process A) = 70%
Calcium recovery (Process B) = 85%
Cyclone residence time to settle 90% solids = 4 min.
Sulfuric acid required to neutralize lime = 30% stoichiometric
Recoverable kiln exhaust heat = 75%
Heating value of wet lignin = 8000 Btu/lbmass

Results

Process A

Cost estimates for lime pretreatment were performed using several different process options. It involves grinding the switchgrass to <40 mesh and mixing with lime, steam, and preheated water. The slurry is held at 120°C for two hours before being pumped through the feed preheater into a series of wash vessels. In the wash and lime recovery train, wash water is filtered from the biomass and contacted with CO_2 to precipitate $CaCO_3$. In our experiments with switchgrass, this method of lime recovery did not work well, but similar experiments with sugarcane bagasse show that about 70% of the lime can be recovered in this fashion using three washings (Nagwani, 1992). (Note: Using ten washings, 86% of the lime was recovered.) After

removing the lime, the biomass is neutralized with sulfuric acid so that it is ready to be sent to the fermentation process. (Note: This step is required only if the fermentor operates below pH 5.5.) The limestone cake is sent to a lime kiln which is fueled with natural gas in Process A-1 and A-2 and with post-fermentation lignin in Process A-3. The heat from the lime kiln is converted to 100-psig steam which can be used in other parts of the plant. In Process A-4, there is no attempt to recover the lime; all of it is neutralized by adding sulfuric acid.

In Process A-1 (Tables 6 and 7), makeup lime (30%) is purchased for $57/tonne and the kiln only converts recovered limestone into lime. In Process A-2 (Tables 8 and 9), makeup lime is made by kilning purchased limestone ($15/tonne) along with the recovered limestone. Because of the high capital required for the kiln and the fact that produced steam barely covers the cost of fuel gas, it appears that it is less expensive to buy the makeup lime. If post-fermentation lignin has no other economical uses and can be recovered for $0.1/MM Btu, it can be used as kiln fuel. Process A-3 (Tables 10 and 11) shows that this option lowers the pretreatment cost to $17/tonne. However, if no lime recovery is attempted, all lime must be purchased and then sulfuric acid must be purchased to neutralize all of the lime. Process A-4 (Tables 12 and 13) shows the cost for this option to be $19.90/tonne grass.

Process B

In each of the previous cases, it is assumed that 70% of the lime can be recovered using CO_2 precipitation. Experiments show that this is not the case for switchgrass, however. An alternative is to send the wash and precipitation filtrate (containing soluble organics, lime, and calcium compounds) to an anaerobic fixed-film digester. The new design involves separating the lime from the biomass using two water washes. The wash effluent is assumed to contain 85% of the lime, 8% of the glucan, 16% of the xylan, 33% of the lignin, and 76% of the protein in the original grass. This effluent is passed through one CO_2-contacting stage and then a fixed-film bio-digester which converts the organics into methane and CO_2. The calcium, which was presumably bound in organic salts, is released and converted into $CaCO_3$. The organic compounds and the limestone slough off of the bed with bacterial sludge. These are filtered to remove water and sent to the lime kiln. The cells and organic substances provide the fuel for the lime kilning process. Excess kiln heat is used to generate steam from which the plant gets credit. Methane from the digester also has heating value.

In order to limit the amount of glucan and xylan which dissolve into the wash effluent, pretreatment must be performed at 100°C rather than at 120°C. This does not cause a noticeable drop in enzymatic digestibility but significantly lowers the amount of soluble glucan and xylan. The key assumption is the lime recovery of 85%. This depends on how much of the organically bound calcium is in solution. Calcium bound in insoluble species cannot be recovered. The bio-digester design equations came from "Anaerobic Treatment of Wastewater in Fixed Film Reactors - A Literature Review" by M. Henze and P. Harremöes (1983).

Figure 2 shows the lime pretreatment process utilizing a methane digester and lime kiln. Table 14 lists the process equipment along with capital and associated utility costs. Not surprisingly, the lime kiln and the fixed-film bio-digester are the most expensive items. Table 15 shows the overall economics of the plant. The processing cost is much lower than for the earlier cases. There is some loss in product quality in that 11% of the fermentable biomass was lost to the wash effluent. This results in a processing cost of $0.12/gallon EtOH which is still considerably lower than $0.17/gallon for the best of the earlier cases. However, most of the protein is lost, so any credits associated with it must also be considered.

Process C

In this process, the washing and filtering equipment of the previous processes is eliminated. In addition, any solubilized organics are not lost to the ethanol fermentation. In Process C, the lime-treated biomass is directly carbonated to neutralize the lime (see Figure 3). Because CO_2 is not a strong acid, the lowest pH that can be achieved is 5.5. This process will not work if the saccharification/fermentation pH must be less than 5.5 since sulfuric acid would react with the calcium to form gypsum; thus it would reduce to Process A-4 with severe penalties to be paid for makeup chemicals and gypsum disposal. Table 16 shows the estimated capital equipment and utility costs. Table 17 shows that the pretreatment cost for Process C is only $7.65/tonne biomass.

Table 6. Lime Make-up with Small Kiln Equipment and Utility Costs for Lime Pretreatment Using CO_2 Contacting and Lime Kiln (Process A-1)

Description	Unit Price	Qty	Lang	M&S Index	Effective Cost	Heat (t steam/t grass)	Electricity (kWh/t grass)
Grinder	70,300	3	4.13	1.0501	914,696		2.24
Pretreat. Tank	84,800	1	4.13	1.1027	386,176		0
Pretreat. Mixer	10,500	1	4.13	1.1027	47,817		0.056
Feed Grass Conv.	5,500	1	4.13	1.1027	25,047		0.02
Feed Lime Conv.	5,500	1	4.13	1.1027	25,047		0.02
Pretr. Slurry Pump	3,500	1	4.13	1.1027	15,939		0.61
Feed Preheat HEX	38,000	1	4.13	1.1027	173,051	0.075	
Wash Vessels	49,043	3	4.13	1.1027	670,020		
Wash V. Mixers	10,000	3	4.13	1.1027	136,619		0.0746
CO_2 Contact Ves.	50,460	3	4.13	1.1027	689,379		
CO_2 Blowers 1-79	8,000	3	4.13	1.6422	162,772		1.49946
Post Wash Cyclo.	56,925	3	4	1.1027	753,224		
Polishing Filters	97,500	3	3	1.1027	967,580		4.2
$CaCO_3$ Filters	97,500	3	3	1.1027	967,580		4.2
Wash & Cont. Pump	16,000	6	4.13	1.1027	437,181		9.57
Wash Grass Conv.	33,000	3	4.13	1.1027	450,842		0.17
$CaCO_3$ Conveyors	9,000	3	4.13	1.1027	122,957		0.06
Lime Kiln	623,000	1	3	1.1027	2,060,862		0.56
Steam HEX	40,000	1	4.13	1.642	271,258	-0.22	

FCI =	Heat=	Elect.=
9,278,046	-0.145	23.28006

Table 7. Lime Make-up with Small Kiln
Equipment and Utility Costs for Lime Pretreatment
Using CO_2 Contacting and Lime Kiln (Process A-1)

	$/tonne grass	cents/gal EtOH	$/yr
FEEDSTOCK			
Grass ($0/tonne)	0.00	0.00	0
Lime Makeup ($57.2/tonne)	1.60	1.60	513,427
Sulfuric Acid ($75/tonne)	2.84	2.84	908,502
UTILITIES			
Cogen. Elect. ($0.02/kWh)	0.47	0.47	148,992
Heat ($4.4/tonne steam)	-0.71	-0.71	-226,896
Nat. Gas ($1.75/MM Btu)	1.00	1.00	320,000
LABOR			
Plant Manager (1/3 @ $90,000/yr)	0.09	0.09	30,000
Supervisors (4/3 @ $70,000/yr)	0.29	0.29	93,333
Workers (13 @ $45,000/yr)	1.83	1.83	585,000
FIXED CHARGES			
Depreciation (0.1 × FCI)	2.90	2.90	927,805
Local Tax (0.03 × FCI)	0.87	0.87	278,341
Insurance (0.007 × FCI)	0.20	0.20	64,946
Maintenance (0.04 × FCI)	1.16	1.16	371,122
Interest (0.15 × 1.15 × FCI)	5.00	5.00	1,600,463
TOTAL	17.55	17.55	5,615,036

Table 8. Limestone Make-up with Large Kiln Equipment and Utility Costs for Lime Pretreatment Using CO_2 Contacting and Lime Kiln (Process A-2)

Description	Unit Price	Qty	Lang	M&S Index	Effective Cost	Heat (t steam/t grass)	Electricity (kWh/t grass)
Grinder	70,300	3	4.13	1.050147	914,696		2.24
Pretreat. Tank	84,800	1	4.13	1.102655	386,176		0
Pretreat. Mixer	10,500	1	4.13	1.102655	47,817		0.056
Feed Grass Conv.	5,500	1	4.13	1.102655	25,047		0.02
Feed Lime Conv.	5,500	1	4.13	1.102655	25,047		0.02
Pretr. Slurry Pump	3,500	1	4.13	1.102655	15,939		0.61
Feed Preheat HEX	38,000	1	4.13	1.102655	173,051	0.075	
Wash Vessels	49,043	3	4.13	1.102655	670,020		
Wash V. Mixers	10,000	3	4.13	1.102655	136,619		0.0746
CO_2 Contact Ves.	50,460	3	4.13	1.102655	689,379		
CO_2 Blowers 1-79	8,000	3	4.13	1.642175	162,772		1.49946
Post Wash Cyclo.	56,925	3	4	1.102655	753,224		
Polishing Filters	97,500	3	3	1.102655	967,580		4.2
$CaCO_3$ Filters	97,500	3	3	1.102655	967,580		4.2
Wash & Cont. Pump	16,000	6	4.13	1.102655	437,181		9.57
Wash Grass Conv.	33,000	3	4.13	1.102655	450,842		0.17
$CaCO_3$ Conveyors	9,000	3	4.13	1.102655	122,957		0.06
Lime Kiln	1,182,000	1	3	1.102655	3,910,014		1.17
Steam HEX	64,000	1	4.13	1.642	434,013	-0.282	

FCI =	Heat=	Elect.=
11,289,954	-0.207	23.89006

Table 9. Limestone Make-up with Large Kiln Equipment and Utility Costs for Lime Pretreatment Using CO_2 Contacting and Lime Kiln (Process A-2)

	$/tonne grass	cents/gal EtOH	$/yr
FEEDSTOCK			
Grass ($0/tonne)	0.00	0.00	0
Lime Makeup ($57.2/tonne)	0.57	0.57	181,946
Sulfuric Acid ($75/tonne)	2.78	2.78	890,332
UTILITIES			
Cogen. Elect. ($0.02/kWh)	0.48	0.48	152,896
Heat ($4.4/tonne steam)	-1.01	-1.01	-323,914
Nat. Gas ($1.75/MM Btu)	1.17	1.17	374,400
LABOR			
Plant Manager (1/3 @ $90,000/yr)	0.09	0.09	30,000
Supervisors (4/3 @ $70,000/yr)	0.29	0.29	93,333
Workers (13 @ $45,000/yr)	1.83	1.83	585,000
FIXED CHARGES			
Depreciation (0.1 × FCI)	3.53	3.53	1,128,995
Local Tax (0.03 × FCI)	1.06	1.06	338,699
Insurance (0.007 × FCI)	0.25	0.25	79,030
Maintenance (0.04 × FCI)	1.41	1.41	451,598
Interest (0.15 × 1.15 × FCI)	6.09	6.09	1,947,517
TOTAL	18.53	18.53	5,929,833

Table 10. Lime Make-up with Small Kiln and Lignin Equipment and Utility Costs for Lime Pretreatment Using CO_2 Contacting and Lime Kiln (Process A-3)

Description	Unit Price	Qty	Lang	M&S Index	Effective Cost	Heat (t steam/t grass)	Electricity (kWh/t grass)
Grinder	70,300	3	4.13	1.050147	914,696		2.24
Pretreat. Tank	84,800	1	4.13	1.102655	386,176		0
Pretreat. Mixer	10,500	1	4.13	1.102655	47,817		0.056
Feed Grass Conv.	5,500	1	4.13	1.102655	25,047		0.02
Feed Lime Conv.	5,500	1	4.13	1.102655	25,047		0.02
Pretr. Slurry Pump	3,500	1	4.13	1.102655	15,939		0.61
Feed Preheat HEX	38,000	1	4.13	1.102655	173,051	0.075	
Wash Vessels	49,043	3	4.13	1.102655	670,020		
Wash V. Mixers	10,000	3	4.13	1.102655	136,619		0.0746
CO_2 Contact Ves.	50,460	3	4.13	1.102655	689,379		
CO_2 Blowers 1-79	8,000	3	4.13	1.642175	162,772		1.49946
Post Wash Cyclo.	56,925	3	4	1.102655	753,224		
Polishing Filters	97,500	3	3	1.102655	967,580		4.2
$CaCO_3$ Filters	97,500	3	3	1.102655	967,580		4.2
Wash & Cont. Pump	16,000	6	4.13	1.102655	437,181		9.57
Wash Grass Conv.	33,000	3	4.13	1.102655	450,842		0.17
$CaCO_3$ Conveyors	9,000	3	4.13	1.102655	122,957		0.06
Lime Kiln	623,000	1	3	1.102655	2,060,862		0.56
Steam HEX	40,000	1	4.13	1.642	271,258	-0.22	

FCI =	Heat=	Steam=
9,278,046	-0.145	23.28006

Table 11. Lime Make-up with Small Kiln and Lignin Equipment and Utility Costs for Lime Pretreatment Using CO_2 Contacting and Lime Kiln (Process A-3)

	$/tonne grass	cents/gal EtOH	$/yr
FEEDSTOCK			
Grass ($0/tonne)	0.00	0.00	0
Lime Makeup ($57.2/tonne)	1.60	1.60	513,427
Sulfuric Acid ($75/tonne)	2.84	2.84	908,502
UTILITIES			
Cogen. Elect. ($0.02/kWh)	0.47	0.47	148,992
Heat ($4.4/tonne steam)	-0.71	-0.71	-226,896
Lignin ($1.75/MM Btu)	0.06	0.06	18,286
LABOR			
Plant Manager (1/3 @ $90,000/yr)	0.09	0.09	30,000
Supervisors (4/3 @ $70,000/yr)	0.29	0.29	93,333
Workers (13 @ $45,000/yr)	1.83	1.83	585,000
FIXED CHARGES			
Depreciation (0.1 × FCI)	2.90	2.90	927,805
Local Tax (0.03 × FCI)	0.87	0.87	278,341
Insurance (0.007 × FCI)	0.20	0.20	64,946
Maintenance (0.04 × FCI)	1.16	1.16	371,122
Interest (0.15 × 1.15 × FCI)	5.00	5.00	1,600,463
TOTAL	16.60	16.60	5,313,322

Table 12. No Lime Recovery
Equipment and Utility Costs for Lime Pretreatment
Using CO_2 Contacting and Lime Kiln (Process A-4)

Description	Unit Price	Qty	Lang	M&S Index	Effective Cost	Heat (t steam/t grass)	Electricity (kWh/t grass)
Grinder	70,300	3	4.13	1.050147	914,696		2.24
Pretreat. Tank	84,800	1	4.13	1.102655	386,176		0
Pretreat. Mixer	10,500	1	4.13	1.102655	47,817		0.056
Feed Grass Conv.	5,500	1	4.13	1.102655	25,047		0.02
Feed Lime Conv.	5,500	1	4.13	1.102655	25,047		0.02
Pretr. Slurry Pump	3,500	1	4.13	1.102655	15,939		0.61
Feed Preheat HEX	38,000	1	4.13	1.102655	173,051	0.075	
Wash Vessels	49,043	3	4.13	1.102655	670,020		
Wash V. Mixers	0	3	4.13	1.102655	0		0
CO_2 Contact Ves.	0	3	4.13	1.102655	0		0
CO_2 Blowers 1-79	0	3	4.13	1.642175	0		0
Post Wash Cyclo.	0	3	4	1.102655	0		0
Polishing Filters	0	3	3	1.102655	0		0
$CaCO_3$ Filters	0	3	3	1.102655	0		0
Wash & Cont. Pump	0	6	4.13	1.102655	0		0
Wash Grass Conv.	0	3	4.13	1.102655	0		0
$CaCO_3$ Conveyors	0	3	4.13	1.102655	0		0
Lime Kiln	0	1	3	1.102655	0		0
Steam HEX	0	1	4.13	1.1027	0		

FCI =	Heat=	Elect.=
2,257,793	0.075	2.946

Table 13. No Lime Recovery
Equipment and Utility Costs for Lime Pretreatment
Using CO_2 Contacting (Process A-4)

	$/tonne grass	cents/gal EtOH	$/yr
FEEDSTOCK			
Grass ($0/tonne)	0.00	0.00	0
Lime Makeup ($57.2/tonne)	5.35	5.35	1,711,424
Sulfuric Acid ($75/tonne)	9.46	9.46	3,028,340
UTILITIES			
Cogen. Elect. ($0.02/kWh)	0.06	0.06	18,854
Heat ($4.4/tonne steam)	0.37	0.37	117,360
Nat. Gas ($1.75/MM Btu)	0.00	0.00	0
LABOR			
Plant Manager (1/3 @ $90,000/yr)	0.09	0.09	30,000
Supervisors (4/3 @ $70,000/yr)	0.29	0.29	93,333
Workers (13 @ $45,000/yr)	1.83	1.83	585,000
FIXED CHARGES			
Depreciation (0.1 × FCI)	0.71	0.71	225,779
Local Tax (0.03 × FCI)	0.21	0.21	67,734
Insurance (0.007 × FCI)	0.05	0.05	15,805
Maintenance (0.04 × FCI)	0.28	0.28	90,312
Interest (0.15 × 1.15 × FCI)	1.22	1.22	389,469
TOTAL	19.92	19.92	6,373,410

Table 14. Lime Make-up with Bio-Digester and Small Kiln Using 1 Stage of CO_2 Contacting Equipment and Utility Costs (Process B)

Description	Unit Price	Qty	Lang	M&S Index	Effective Cost	Heat (t steam/t grass)	Electricity (kWh/t grass)
Grinder	70,300	3	4.13	1.050147	914,696		2.24
Pretreat. Tank	84,800	1	4.13	1.102655	386,176		0
Pretreat. Mixer	10,500	1	4.13	1.102655	47,817		0.056
Feed Grass Conv.	5,500	1	4.13	1.102655	25,047		0.02
Feed Lime Conv.	5,500	1	4.13	1.102655	25,047		0.02
Pretr. Slurry Pump	3,500	1	4.13	1.102655	15,939		0.61
Feed Preheat HEX	38,000	1	4.13	1.102655	173,051	0.075	
Wash Vessels	49,043	2	4.13	1.102655	446,680		
Wash V. Mixers	10,000	2	4.13	1.102655	91,079		0.0746
CO_2 Contact Ves.	50,460	1	4.13	1.102655	229,793		
CO_2 Blowers 1-79	8,000	1	4.13	1.642175	54,257		1.49946
Post Wash Cyclo.	56,925	2	4	1.102655	502,149		
Polishing Filters	97,500	2	3	1.102655	645,053		4.2
$CaCO_3$ Filters	97,500	2	3	1.102655	645,053		4.2
Wash & Cont. Pump	16,000	3	4.13	1.102655	218,590		9.57
Wash Grass Conv.	33,000	2	4.13	1.102655	300,562		0.17
$CaCO_3$ Conveyors	9,000	1	4.13	1.102655	40,986		0.06
Digester	443,000	1	4.13	1.102655	2,017,407		
Lime Kiln	760,000	1	3	1.102655	2,514,053		0.68
Steam HEX	90,000	1	4.13	1.642	610,331	-0.656	

FCI=	Heat=	Steam=
9,903,766	-0.581	23.40006

Table 15. Lime Make-up with Small Kiln and Bio-Digester Using 1 Stage of CO_2 Contacting (Process B)

	$/tonne grass	cents/gal EtOH	$/yr
FEEDSTOCK			
Grass ($0/tonne)	0.00	0.00	0
Lime Makeup ($57.2/tonne)	0.80	0.90	256,714
Sulfuric Acid ($75/tonne)	1.42	1.59	454,251
UTILITIES			
Cogen. Elect. ($0.02/kWh)	0.47	0.53	149,760
Heat ($4.4/tonne steam)	-2.84	-3.19	-909,149
Nat. Gas ($1.75/MM Btu)	-2.30	-2.58	-736,000
LABOR			
Plant Manager (1/3 @ $90,000/yr)	0.09	0.11	30,000
Supervisors (4/3 @ $70,000/yr)	0.29	0.33	93,333
Workers (13 @ $45,000/yr)	1.83	2.05	585,000
FIXED CHARGES			
Depreciation (0.1 × FCI)	3.09	3.48	990,377
Local Tax (0.03 × FCI)	0.93	1.04	297,113
Insurance (0.007 × FCI)	0.22	0.24	69,326
Maintenance (0.04 × FCI)	1.24	1.39	396,151
Interest (0.15 × 1.15 × FCI)	5.34	6.00	1,708,400
TOTAL	10.58	11.89	3,385,276

Table 16. Direct Carbonation Equipment and Utility Costs (Process C)

Description	Unit Price	Qty	Lang	M&S Index	Effective Cost	Heat (t steam/t grass)	Electricity (kWh/t grass)
Grinder	70,300	3	4.13	1.050147	914,696		2.24
Pretreat. Tank	84,800	1	4.13	1.102655	386,176		0
Pretreat. Mixer	10,500	1	4.13	1.102655	47,817		0.056
Feed Grass Conv.	5,500	1	4.13	1.102655	25,047		0.02
Feed Lime Conv.	5,500	1	4.13	1.102655	25,047		0.02
Slurry Pump	3,500	2	4.13	1.102655	31,878		1.24
Feed Preheat HEX	38,000	1	4.13	1.102655	173,051	0.075	
CO_2 Blower	8,000	3	4.13	1.642175	162,772		1.49946
Carbonation Tank	84,800	1	4.13	1.102653	386,176		
Lime Kiln	850,000	1	3	1.102655	2,811,770		0.76
Steam HEX	40,000	1	4.13	1.642	271,258	-0.22	

FCI=	Heat=	Elect.=
5,235,688	-0.145	5,835

Table 17. Direct Carbonation (Process C)

	$/tonne grass	cents/gal EtOH	$/yr
FEEDSTOCK			
Grass ($0/tonne)	0.00	0.00	0
Lime Makeup ($57.2/tonne)	0.27	0.27	86,400
Sulfuric Acid ($75/tonne)	0.00	0.00	0
UTILITIES			
Cogen. Elect. ($0.02/kWh)	0.11	0.11	33,680
Heat ($4.4/tonne steam)	-0.71	-0.71	-226,896
Lignin ($1.75/MM Btu)	0.06	0.06	18,286
LABOR			
Plant Manager (1/3 @ $90,000/yr)	0.09	0.09	30,000
Supervisors (4/3 @ $70,000/yr)	0.29	0.29	93,333
Workers (13 @ $45,000/yr)	1.83	1.83	585,000
FIXED CHARGES			
Depreciation (0.1 × FCI)	1.64	1.64	523,569
Local Tax (0.03 × FCI)	0.49	0.49	157,070
Insurance (0.007 × FCI)	0.11	0.11	36,650
Maintenance (0.04 × FCI)	0.65	0.65	209,427
Interest (0.15 × 1.15 × FCI)	2.82	2.82	903,156
TOTAL	7.65	7.65	2,449,675

Conclusions

Process A is desirable if lime removal from the pretreated biomass is high, little biomass is solubilized during pretreatment, and lime is readily precipitated upon carbonation. It gives complete freedom in the choice of saccharification/fermentation pH. Of the various options explored, it appears to be the most expensive. The best process option (A-3) costs $16.60/tonne.

Process B is desirable if lime removal from the pretreated biomass is high, but so much biomass is solubilized that it prevents lime from being readily precipitated upon carbonation. The solubilized biomass is converted to methane and carbon dioxide which frees the calcium to be recovered as calcium carbonate. This process also gives complete freedom in the choice of saccharification/fermentation pH. It appears to be less expensive than Process A (about $10.58/tonne), but some of the sugars are lost to methane production which reduces alcohol yields.

Process C is necessary if lime removal from the pretreated biomass is low. The lime-treated biomass is neutralized directly by contacting it with CO_2. Because CO_2 is a weak acid, the lowest attainable pH is 5.5; thus, the saccharification/fermentation is restricted to a pH above 5.5. This is not a severe restriction since many bacterial and yeast fermentations operate above this pH. Because this process is so simple, it has the lowest cost, $7.65/tonne.

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Appendix — Sample Calculations

G-1 Grinder

Laboratory tests showed that lime pretreatment worked well with switchgrass ground to -40 mesh. To achieve this particle size before pretreatment, the grass should be ground in a burr mill which is a robust and efficient version of the classic stone mills used to grind grain. In the stone mills, a stone disc rotates against a stationary disc. The feed is introduced through a hole in the center of the disc. The discs are closely spaced so that so that the feed is ground as it moves between the discs.

In a modern burr mill, the stone discs are replaced with steel plates made of "Ni-hard," an extremely hard cast-iron alloy with a high nickel content. The steel plates have a waffle texture so the feed material is cut as the mobile plate rotates. Adjusting the clearance between the plates determines the particle size of the product. Particles of 200 mesh are possible with 40 mesh easily achieved. The burr mill is tolerant of sand and grit that is likely to accompany the biomass feed. Sand in the feed does cause greater wear of the steel plates.

Burr mills are manufactured by Winona Attrition Mill Company, 1009 West Fifth Street, Winona, MN 55987 (Telephone (507) 452-2716). Howard Tomashek, a technical representative of the company, recommends the Model 3086 burr mill. It has a 30" diameter and processes 25 tons/h. The mobile plate rotates at 1800 rpm and consumes 50 hp. The steel plates must be replaced every 6-8 months at a cost of $400 for the entire machine. A magnetic separator is an optional feature that helps exclude ferrous metals from the burr mill.

To ensure a uniform feed particle size and to protect the burr mill from damaging trash, Howard Tomashek recommends a "crusher feeder and lump breaker" to process the feed that enters the burr mill. He recommends the Model WA-22. It requires a 10 hp motor and processes 25 tons/h. It features a shear pin that stops the machine if damaging trash is introduced.

Two grinders will be able to process 50 tons/h of switchgrass. Since our process has 40 tonnes/h (44 tons/h), two are sufficient. However, since grinders are likely to require frequent maintenance either for replacing the burr plates or to replace the shear pin if damaging trash is contained in the feed, a spare grinding system will be available for service.

Model 3086 Burr Mill =	$16,895	
Motor =	$3000	(50-hp, TEEP, 3 phase, 230 V)
Magnetic Separator =	$600	
Hopper Agitator =	$400	
Model WA-22 =	$1,438	
Motor Mount =	$300	
Motor =	$800	(10-hp, Enclosed fan-cooled)
Total =	$23,433	
Quantity = 3		

A1-Grass Feed Auger

A 16-in diameter screw conveyor can move 250 ton/h of sand, so this should be sufficient.

Cost = $10,000 (20-ft long, 3-hp)

$$Energy = 2 \times \frac{60\ hp{\cdot}h}{36.5 tonne} \times \frac{0.746\ kW}{hp} = 2.45\ \frac{kWh}{tonne}$$

Quantity = 1

$$Energy = \frac{3\ hp{\cdot}h}{40\ tonne} \times \frac{0.746\ kW}{hp} = 0.056\ \frac{kWh}{tonne}$$

V1-Pretreatment Tank

During pretreatment, the ground grass is mixed with 9 tonnes H_2O/tonne dry grass and 0.1 tonnes $Ca(OH)_2$ (lime)/tonne dry grass. The contents are held at 120°C for 2 hours. Moisture content of grass is 6.7% H_2O. So for a grass rate of 40 tonnes/hour, the rates of dry grass, water, and lime are 37.32, 336, and 3.73 tonnes/hour respectively. Total mass flow is 377 tonnes/hour.

Assume a 2-h residence time.

$$Working\ Volume = \frac{377\ tonne}{h} \times 2\ h \times \frac{1\ m^3}{tonne} = 754\ m^3$$

$$V = Actual\ Volume = 1.15\ (Working\ Volume) = 1.15\ (754\ m^3) = 867\ m^3$$

$$D = \sqrt[3]{\frac{4V}{\pi}} = \sqrt[3]{867\ m^3\ \frac{4}{\pi}} = 10.33\ m$$

$$A = 2(\frac{\pi}{4}D^2) + (\pi D)D = \frac{3\pi}{2}D^2 = \frac{3\pi}{2}(10.33\ m)^2 = 503\ m^2$$

$$Cost = \frac{\$168}{m^2}(503\ m^2) = \$84{,}477$$

$$Quantity = 1$$

M1 - Pretreatment Mixer

$$Power = \frac{3\ W}{m^3} \times 867\ m^3 \times \frac{hp}{746\ W} = 3.49\ hp$$

Agitator with motor = $10,500 (3 hp, stainless steel, dual impeller)

Quantity = 1

$$Energy = \frac{2\ hp{\cdot}h}{40\ tonne} \times \frac{0.746\ kW}{hp} = 0.056\ \frac{kWh}{tonne}$$

P1 - Pretreatment Slurry Pump

$$Flow = \frac{377\ tonne}{h} \times \frac{1\ m^3}{tonne} \times \frac{7.481\ gal}{ft^3} \times \frac{h}{60\ \text{min}} = \frac{1660\ gal}{\text{min}}$$

$\Delta P = 20\ psi\ (estimate)$

$$Capacity\ Factor = \frac{1660\ gal}{\text{min}} \times 20\ psi = \frac{33{,}200\ gal{\cdot}psi}{\text{min}}$$

$Cost$ = \$3,500 (*AVS, chemical, horizontal, ductile iron casing up to 150 psi*)

$$Power = \frac{1660\ gal}{\text{min}} \times \frac{20\ lbf}{in^2} \frac{144\ in^2}{ft^2} \times \frac{ft^3}{7.48\ gal} \times \frac{\text{min}}{60\ s}$$

$$\times \frac{kW{\cdot}s}{737\ ft{\cdot}lbf} \times \frac{1}{0.6} = 24.1\ kW$$

$$Energy = \frac{24.1\ kW{\cdot}h}{40\ tonne} = \frac{0.61\ kW{\cdot}h}{tonne}$$

HE-1 Feed Pre-Heat Exchanger

Water (27°C) entering the pretreatment vessel is preheated to 112°C in a heat exchanger using heat from the 120°C slurry being pumped out of the vessel. Extra heat needed to raise the water, grass, and lime temperatures to 120°C is supplied by live steam fed directly to the pretreatment vessel. When the steam condenses, the condensate is added to the slurry. The slurry leaves the heat exchanger at 38°C.

$$C_p(m) = \frac{1}{377\ tonne\ slurry} \times \left(\frac{1782\ Btu}{tonne\ ^oC} \times 333\ tonne\ H_2O + \frac{1121\ Btu}{tonne\ ^oC}\right.$$

$$\left. \times\ 3.73\ tonne\ lime + \frac{398\ Btu}{tonne\ ^oC} \times 40\ tonne\ grass\right)$$

F_m = *slurry flow rate* = 377 *tonne/h*

F_s = 100 *psig steam flow (tonne/h)*

F_f = 27°C *feed water flow (tonne/h)*

Q_s = *heat supplied by steam (Btu/h)*

Q_x = *heat transferred to feed water (Btu/h)*

T_h = *temperature of water exiting heat exchanger (°C)*

$Q_t = Q_s + Q_x$ = *total heat supplied to material entering vessel*

$$Q_x = F_f \times \frac{1782\ Btu}{tonne\ ^oC} \times (T_h - 27)^oC$$

$$Q_s = F_s \times \frac{(1190 - 217)\ Btu}{lbm} \times \frac{2200\ lbm}{tonne}$$

$$Q_s = \frac{40\ tonne}{h} \times \frac{398\ Btu}{tonne\ ^oC} \times (120 - 27)^oC$$

$$+ \frac{3.7\ tonne}{h} \times \frac{1121\ Btu}{tonne\ ^oC} \times (120 - 27)^oC$$

$$+ \frac{F_f\ tonne}{h} \times \frac{1782\ Btu}{tonne\ ^oC} \times (120 - T_h)^oC$$

$$F_f + F_s = 336\ tonne/h$$

Solving for Q_x, F_s, F_f, and T_h Yields:

Q_x = 50,760,000 Btu/h
F_s = 2.98 tonne steam/h
F_f= 333 tonne feed water/h
T_h= 112°C

Heat exchanger surface area, A, is found from:

$$A = \frac{Q_x}{U\ \Delta T_{ln}}$$

$$U = \frac{540\ Btu}{ft^2\ h\ ^oC}$$

$$\Delta T_{ln} = \frac{(38 - 27) - (120 - 112)}{\ln \frac{38 - 27}{120 - 112}} = 9.4^oC$$

$$\therefore\ A = \frac{50{,}760{,}000}{540 \times 9.4} = 6{,}290\ ft^2$$

Cost = \$38,000 (*steel*, 1-1/4$^{''}$ *tubes*, *tri-pitch*, 16$^{'}$ *long*)

$$Steam\ Usage = \frac{2.98\ tonne}{h} \times \frac{h}{40\ tonne\ grass} = \frac{0.075\ tonne\ steam}{tonne\ grass}$$

V2, V3, V4 - Wash Vessels

Assume residence time is 0.5 h. Slurry is diluted with 243 tonnes water per hour to aid in lime solvation. Experiments using pretreated bagasse show that 70% of the lime can be recovered with three wash and precipitation steps.

$$F = \frac{380\ tonne\ slurry}{h} + \frac{243\ tonne\ H_2O}{h} = \frac{623\ tonne\ wash\ slurry}{h}$$

$$F = 623\ m^3/h$$

$$Working\ Volume = 1.15\ F\ \tau = 1.15\ x\ \frac{623\ m^3}{h}\ x\ 0.5\ h = 359\ m^3/h$$

$$D = \sqrt[3]{\frac{4}{\pi}\ 359} = 7.7\ m$$

$$A = 1.5\ \pi\ D^2 = 1.5 \times \pi \times (7.7\ m)^2 = 280\ m^2$$

$$Wash\ vessel\ cost = (280\ m^2) \times (\$168/m^2) = \$47{,}000\ each$$

$$Quantity = 3$$

$$Power = \frac{3\ W}{m^3} \times \frac{312\ m^3}{h} \times \frac{hp}{746\ W} = 1.25\ hp$$

$$Mixer\ cost = \$10{,}000\ ea.\ for\ 2\text{-}hp,\ stainless\ steel,\ dual\ impeller$$

$$Quantity = 3$$

$$Energy = \frac{1.25\ hp{\cdot}h}{40\ tonne} \times 3 \times \frac{0.746\ kW}{hp} = 0.07\ kWh/tonne$$

C1, C2, C3 - Hydrocyclones

Assume 4 min. residence time.

$$Volume = V = 4\ min \times \frac{623\ m^3}{h} \times \frac{h}{60\ min} = 41.5\ m^3$$

$$D = \sqrt[3]{\frac{16}{5\pi} V} = 3.48\ m$$

$$Mass = M = \frac{0.283\ lb}{in^3} \times 1.81 \times \pi \times 0.5\ in$$

$$(3.48\ m \times \frac{ft}{0.3048\ m} \times \frac{12\ in}{ft})^2 = 15{,}140\ lb$$

$$M_{TOT} = 1.2 \times 15{,}140\ lb = 18{,}200\ lb$$

$$Cost = 80 \times (18{,}200)^{0.66} = \$55{,}300$$

Quantity = 3.

Inlet Velocity and Pressure Drop

$$Velocity = v = \sqrt{2\ g\ r}$$

$$v = \sqrt{2 \times \frac{9.8\ m}{s^2} \times \frac{3.5\ m}{2}} = 8.3\ m/s$$

$$\Delta P = \frac{1000\ kg}{m^3} \times 0.5 \times (\frac{8.3\ m}{s})^2 \times \frac{14.7\ psi}{101325\ Pa} = 4.98\ psi$$

F1, F2, F3 - Polishing Filters

Assume 0.5% solids; 13 lb/ft^3 solids density in cake; 20% solids.

$$Solids\ Load = \frac{623\ tonne}{h} \times \frac{0.005\ tonne\ solid}{tonne\ liquid} \times \frac{2200\ lb}{tonne}$$

$$\times \frac{h}{60\ min} \times \frac{ft^3}{13\ lb} = 8.8\ ft^3/min$$

10" bowl in centrifugal Filter is acceptable. Requires 75 hp motor and drive.

Filter = $75,000
Motor = $4,500
Drive = $18,000

Total = $97,500

Quantity = 3

V5, V6, V7 - Precipitation Vessels

Filtrate from the wash vessels laden with dissolved lime is contacted with CO_2 in the precipitation vessels. $CaCO_3$ precipitates and is subsequently filtered and converted to lime in the lime kiln.

$$CO_2\ demand = \frac{3.74\ tonne\ Ca(OH)_2}{623\ m^3} \times \frac{1000\ kg}{tonne} \times \frac{kg\text{-}mole\ Ca(OH)_2}{74\ kg\ Ca(OH)_2}$$

$$\times \frac{kg\text{-}mole\ CO_2}{kg\text{-}mole\ Ca(OH)_2} = \frac{0.081\ kg\text{-}mole\ CO_2}{m^3}$$

$$\eta_{CO_2} = \frac{0.081\ kg\text{-}moles\ CO_2}{\tau\ h \cdot m^3} = H\ k_L a\ (\bar{p} - p*)$$

$$Assume:\ \bar{p} = 1\ atm;\ p* = 0\ atm;\ H = \frac{0.022\ kg\text{-}mole\ CO_2}{m^3 \cdot atm}$$

$$k_L a = \frac{0.081\ kg\text{-}mole\ CO_2}{0.5\ h \cdot m^3} \times \frac{m^3 \cdot atm}{0.022\ kg\text{-}mole\ CO_2} \times \frac{1}{1\ atm} = 7.36\ h^{-1}$$

(from Fig. 6.13, Aiba, 1973): $P_v = 0.065\ hp/m^3$

$$P_{aer} = 3\ P_v\ F\ \tau$$

$$P_{aer} = 3 \times \frac{0.065\ hp}{m^3} \times \frac{623\ m^3}{h} \times 0.5\ h \times \frac{0.745\ kW}{hp} \times \frac{h}{40\ tonne}$$

$$P_{aer} = 1.13\ kWh/tonne$$

$$Working\ Volume = 1.15\ F\ \tau = 1.15 \times \frac{623\ m^3}{h} \times 0.5\ h = 359\ m^3/h$$

$$D = \sqrt[3]{\frac{4}{\pi}\ 359} = 7.7\ m$$

$$A = 1.5\ \pi\ D^2 = 1.5 \times \pi \times (7.7\ m)^2 = 280\ m^2$$

$$Precipitation\ vessel\ cost = (280\ m^2) \times (\$168/m^2) = \$47{,}000\ each$$

$$Quantity = 3$$

CO_2 Blowers

$$F_{CO_2} = \frac{0.081\ kg\text{-}mole\ CO_2}{m^3} \times \frac{2.2\ lb\text{-}mole}{kg\text{-}mole} \times \frac{359\ ft^3}{lb\text{-}mole}$$

$$\times \frac{623\ m^3}{h} \times \frac{h}{60\ \text{min}} = \frac{664\ ft^3\ CO_2}{\text{min}}$$

Blower Cost = $12,000 each (10 psi, rotary)

F4, F5, F6 - $CaCO_3$ Filters

Assume solids density is 13 lb/ft^3; 20% solids.

$$CaCO_3\ load = \frac{3.73\ tonne\ Ca(OH)_2}{h} \times \frac{100\ mole\ CaCO_3}{74\ mole\ Ca(OH)_2}$$

$$\times \frac{0.7\ tonne\ recovered}{tonne} \times 2\ safety\ factor \times 3\ filters$$

$$CaCO_3\ load = 2.35\ tonne/h$$

$$Solids\ content = \frac{2.35\ tonne}{623\ m^3} \times \frac{1000\ kg}{tonne} = 3.78\ kg/m^3 = 0.35\%\ solids$$

$$Solids\ load = \frac{623\ tonne}{h} \times \frac{0.0035\ tonne\ solid}{tonne\ liquid} \times \frac{2200\ lb}{tonne} \times \frac{h}{60\ min}$$

$$\times \frac{ft^3}{13\ lb} = 6.15\ ft^3/min$$

40-in diameter bowl centrifugal filter is acceptable. Requires a 75-hp motor and drive.

Filter = $75,000
Motor = $4,500
Drive = $18,000

Total = $97,500

Quantity = 3

Energy for 6 filters:

$$Energy = 6 \times \frac{75\ hp{\cdot}h}{40\ tonne} \times \frac{0.746\ kW}{hp} = 8.4\ kWh/tonne$$

P2-P7 - Wash and Precipitation Vessel Pumps

$$Q = 2\ safety\ factor \times \frac{623\ tonne}{h} \times \frac{h}{60\ min} \times \frac{m^3}{tonne}$$

$$\times \left(\frac{ft}{0.3048\ m}\right)^3 \times \frac{7.48\ gal}{ft^3} = 5{,}470\ gal/min$$

Pressure drop (ΔP) = 30 psi to account for filters and cyclones.

$$Capacity\ factor = \frac{5{,}470\ gal}{min} \times 30\ psi = \frac{164{,}000\ gal \cdot psi}{min}$$

Cost = $16,000 each. (API 610 cast steel up to 150 psi).

Quantity = 6.

$$Power = \frac{2735\ gal}{min} \times \frac{144 in^2}{ft^2} \times \frac{30\ lbf}{in^2} \times \frac{ft^3}{7.48\ gal} \times \frac{min}{60\ s}$$

$$\times \frac{kWs}{737\ ft \cdot lbf} \times \frac{1}{0.6} \times 6 = 357\ kW$$

$$Energy = \frac{357\ kWh}{40\ tonne} = \frac{8.93\ kWh}{tonne\ grass}$$

A2, A3, A4 - Washed Grass Conveyors

Assume washed cake is 35% solids and conveyor length is 30 feet.

$$Q = \frac{40\ tonne\ grass}{h} \times 2\ safety\ factor\ \frac{tonne\ cake}{0.35\ tonne\ grass} = \frac{228\ tonne}{h}$$

Cost = $11,000 each.

Quantity = 3.

$$Energy = \frac{3\ hp{\cdot}h}{40\ tonne} \times \frac{0.746\ kW}{hp} \times 3 = \frac{0.17\ kWh}{tonne\ grass}$$

K1 - Lime Kiln

Recovered limestone cake having 50% water is converted to lime in a kiln operating at 900°C which is fueled with natural gas. Steam is made by heat exchanging the exhaust kiln gases with 100-psig condensate. Assume 75% of the kiln energy can be utilized for steam production.

$$Kiln\ Capacity = \frac{3.73\ tonne\ Ca(OH)_2}{h} \times \frac{100\ tonne\ CaCO_3}{74\ tonne\ Ca(OH)_2}$$

$$\times \frac{0.7\ tonne\ recovered}{tonne} \times \frac{1.1\ ton}{tonne} \times \frac{24\ h}{day} = 93.4\ ton\ CaCO_3/day$$

Cost = $623,000 for 8' x 140' kiln (Perry, Table 20-16, 1984)

$$Drive\ Energy = \frac{30\ hp{\cdot}h}{tonne} \times \frac{0.744\ kW}{hp} = \frac{0.56\ kWh}{tonne\ grass}$$

Kiln Heat Requirements

Heat needed to operate the kiln is the sum of the heats needed to heat the limestone and water to 900°C, to heat nitrogen and oxygen in fuel mixture to 900°C, and to react limestone into lime.

$$q_{tot} = q_{H_2O} + q_{CaCO_3} + q_{rxn}$$

Necessary Thermodynamic Data:

Heat Capacities:

Compound	Mw	Cp (cal/gmole K)	Reference
$H_2O_{(l)}$	18	1	(Table 3-181, Perry, 1984)
$H_2O_{(g)}$	18	$8.22 + 0.00015T + 0.00000134T^2$	(Table 3-181, Perry, 1984)
$CaCO_{3(s)}$	100	$19.68 + 0.01189T - 307600/T^2$	(Table 3-181, Perry, 1984)
$CaO_{(s)}$	56	$10 + 0.00484T - 108000/T^2$	(Table 3-181, Perry, 1984)
$CO_{2(g)}$	44	$10.34 + 0.00274T - 195500/T^2$	(Table 3-181, Perry, 1984)

Heat of Vaporization of H_2O = 9690 cal/gmole (Table 3-237, Perry, 1984)

ΔH_{rxn} ($CaCO_3 \rightarrow CaO + CO_2$) = 43.8 kcal/gmole (Table 3-206, Perry, 1984)

$$q_{H_2O} = Mw\ \dot{m}_w\ (Cp_l(100-38)^oC + \Delta H_v + \int_{373}^{1173} Cp_g\ dT) \times \frac{9.486\times10^{-4}\ Btu}{0.239\ cal}$$

$$q_{H_2O} = 13.7\times10^6\ Btu/h$$

$$\dot{m}_w(CaCO_3) = \frac{3.54\ tonne}{h} \times \frac{1000\ kg}{tonne} \times \frac{1000\ g}{kg} = 3.54\times10^6\ g/h$$

$$q_{CaCO_3} = Mw\ \dot{m}_w \int_{311}^{1173} Cp_s\ dT = 3.35\times10^6\ Btu/h$$

$$q_{rxn} = Mw\ \dot{m}_{CaCO_3} \left(\int_{1173}^{298} Cp_{CaCO_3}\ dT + \Delta H_{rxn} + \int_{298}^{1173} Cp_{CO_2}\ dT \right.$$

$$\left. + \int_{298}^{1173} Cp_{CaO}\ dT \right) \times \frac{9.486x10^{-4}\ Btu}{0.239\ cal} = 5.84\times10^6\ Btu/h$$

$$q_{tot} = (13.7 + 3.4 + 5.8)\times10^6\ Btu/h = 22.9\times10^6\ Btu/h$$

Gas unit price = \$1.75/MM Btu

$$Gas\ Cost = 22.9\times10^6\ \frac{Btu}{h} \times \frac{\$1.75}{MM\ Btu} \times \frac{h}{tonne\ grass} = \$1/tonne\ grass$$

HE-2 Kiln Heat Exchanger

Assume 75% of the kiln heat can be used to produce 100-psig steam in a boiler (Perry, 20-37, 1984).

$$q_{avail} = 0.75 \times 22.9 \frac{MM\ Btu}{h} = 17.2 \frac{MM\ Btu}{h}$$

$$\dot{m}_{steam} = 17.2 \frac{MM\ Btu}{h} \times \frac{lbm\ steam}{(1187 - 298)\ Btu} = 19{,}300 \frac{lbm\ steam}{h}$$

$$Steam\ credit = 19{,}300 \frac{lbm\ steam}{h} \times \frac{tonne}{2200\ lbm} \times \frac{\$4.9}{tonne}$$

$$\times \frac{h}{40\ tonne} = \$1.08/tonne\ grass$$

$$q_{avail} = UA\Delta T_{ln}$$

$$U = 10 \frac{Btu}{h \cdot ft^2 \cdot {}^\circ F}$$

$$\Delta T_{ln} = \frac{(900-170) + (300-170)}{\ln \frac{900-170}{300-170}} = 498^\circ C = 897^\circ F$$

$$A = \frac{q}{U\Delta T_{ln}} = \frac{17.2 \times 10^6\ Btu/h}{10 \frac{Btu}{h \cdot ft^2 \cdot {}^\circ F} \times 897^\circ F} = 1900\ ft^2$$

Steam boiler is made of high-alloy metallurgy which doubles the cost over a carbon steel version. The 1900-ft^2 heat exchanger with 1-in x 1-in pitch and 16-ft bundles costs $40,000.

BD-1 Anaerobic Fixed-Film Bio-Digester

Because experiments showed that not very much of the calcium used to pretreat switchgrass was recoverable using CO_2 precipitation, an alternative case using a methane digester was studied. In this case, the biomass slurry is filtered and the filtrate is passed through an anaerobic fixed-film reactor. The filtrate contains low concentrations of soluble xylan, glucan, proteins, and lignin, along with dissolved $Ca(OH)_2$ and complexed calcium. In the fixed-film reactor, these compounds diffuse into the bacterial film attached to the reactor's packing material. The glucan, xylan, proteins, and calcium complexes are reacted into methane (CH_4) and CO_2. The CO_2 combines with the calcium to form $CaCO_3$. The lignin passes through without reacting. In these reactors, the bacterial film sloughs off the packing but has a much longer residence time than the liquid. Methane and excess CO_2 escape out of the top of the reactor. The methane is collected and used as fuel. Effluent (water, lignin, $CaCO_3$, and bacterial slough) are contacted with CO_2 to precipitate any remaining $Ca(OH)_2$ and filtered. The water is recycled to the lime pretreater and the cake is kilned to make lime and heat for steam production.

Since it is largely cellulosic, the biomass oxidation reaction can be approximated as:

$$CH_2O + O_2 \rightarrow CO_2 + H_2O$$

There are 32 kg O_2 consumed for 30 kg of biodegradable solids.

$$COD\ (chem.\ O_2\ demand) = 7.4\ \frac{tonne\ BD\ solids}{h} \times \frac{32\ tonne\ O_2}{30\ tonne\ BD\ solids}$$

$$COD = 7.9\ tonne/h = 190{,}000\ kg/d$$

Suggested conditions for methane production at 35°C are solids residence time, θ_x, of 15 days and biodegradable loading, B_x, of 0.8 kg COD/kg VSS/day. (VSS is volatile suspended solids.) For solids concentration, X_2, of 30 kg VSS/m^3, the volumetric biodegradable load, B_v, is 21 kg COD/m^3/day as shown in Fig. 7.1 in Henze and Harremöes (1983). The tank volume, V is:

$$V = 190{,}000\ \frac{kg\ COD}{d} \times \frac{m^3 \cdot d}{21\ kg\ COD} = 9050\ m^3$$

$$V_{act} = 1.15 \times 9050\ m^3 = 10{,}400\ m^3$$

$$D = \sqrt[3]{\frac{4 \times 10{,}400}{\pi}} = 23.7\ m$$

$$Area = \frac{3\pi}{2} \times (23.7\ m)^2 = 2640\ m^2$$

$$Reactor\ Cost = 2640\ m^2 \times \frac{\$168}{m^2} = \$443{,}000$$

Assume biomass conversion is 92% and methanogenation reaction occurs as:

$$CH_2O \rightarrow \frac{1}{2} CH_4 + \frac{1}{2} CO_2$$

$$\dot{m}_{CH_4} = 0.92 \times \frac{7.4\,tonne\,VSS}{h} \times \frac{tonne\ mole\ VSS}{30\,tonne\,VSS} \times \frac{0.5\,tonne\ mole\,CH_4}{tonne\ mole\,VSS}$$

$$\times \frac{16\,tonne\,CH_4}{tonne\ mole}$$

$$\dot{m}_{CH_4} = 1.82 \frac{tonne\,CH_4}{h}$$

$$CH_4 credit = \frac{1.82\,tonne\,CH_4}{h} \times \frac{2200\,lbm}{tonne} \times \frac{23{,}000\,Btu}{lbm\,CH_4} \times \frac{\$1.00}{10^6\,Btu}$$

$$\times \frac{h}{40\,tonne\,grass}$$

$$CH_4 credit = \frac{\$2.3}{tonne\,grass}$$

Decrease in Biomass Value

Since the bacterial slough and lignin are sent to the kiln, they are being used as fuel and such credit is applied to them. However, they are no longer in the SSF biomass and therefore cause a decrease in fermentation or by-product value of the SSF biomass. Essentially, some valuable xylan and glucan are lost from the SSF biomass and this can be determined from the assay of switchgrasses soluble fraction. Switchgrass is composed of 20% xylan and 33.8% glucan. After pretreatment, 16% of the xylan and 7.3% of the glucan are soluble. The soluble portions are sent to the bio-digester and kiln and are therefore not available for SSF.

$$\textit{Original digestibility} = 20\% + 33.8\% = 53.8\%$$

$$\textit{Post wash digestibility} = 20\% \times (1 - .16) + 33.8\% \times (1 - .073) = 48.1\%$$

$$\textit{Post wash value} = \frac{48.1}{53.8} \times 100\% = 89\%$$

The decrease in biomass value to SSF fermentation is reflected in economic calculations for the cost of pretreatment per gallon of ethanol produced.

K1 - Lime Kiln in Bio-Digester Option

The lime kiln needed for the bio-digester option differs significantly from the Case I lime kiln. Primarily, the bacterial slough and lignin are filtered along with the limestone cake which is sent to the kiln. The organic slough and lignin serve as fuel for the kiln operation and save on natural gas costs. Also, excess heat is produced by the kiln which produces more steam than in Case I. Entering the kiln is a wet filtercake containing limestone, unconverted VSS, lignin, and water.

$$\dot{m}_{CaCO_3} = \frac{32\ \ tonne\,Ca(OH)_2}{h} \times \frac{100\ \ tonne\,CaCO_3}{74\ \ tonne\,Ca(OH)_2} = 4.3\,\frac{tonne\,CaCO_3}{h}$$

$$Kiln\ capacity = \frac{4.3\ \ tonne\,CaCO_3}{h} \times \frac{1.1\ \ ton}{tonne} \times \frac{24\ \ h}{d} = 114\,\frac{ton}{d}$$

$$\dot{m}_{lignin} = 2.7\,\frac{tonne}{h}$$

$$\dot{m}_{VSS} = 0.08x\,\frac{7.4\ \ tonne\,total\,VSS}{h} = 0.6\,\frac{tonne\,VSS}{h}$$

$$\dot{m}_{H_2O} = \dot{m}_{CaCO_3} + \dot{m}_{lignin} + \dot{m}_{VSS} = 7.6\,\frac{tonne\ H_2O}{h}$$

$$\dot{m}_{fuel} = \dot{m}_{lignin} + \dot{m}_{VSS} = 3.3\,\frac{tonne\,fuel}{h}$$

According to Perry's (Table 20-16, 1984), the equipment cost for a 114 ton/d lime kiln is $760,000 and the drive energy required to turn the kiln costs $0.68/tonne grass.

Kiln Heat Requirements

The kiln requires enough heat to raise the limestone and water temperatures from 25°C to 900°C and the heat of reaction in the limestone to lime conversion. This heat is provided by combustion of the lignin and unconverted VSS. The biomass can be assumed to have a heating value of 8000 Btu/lbm (Table 9-8, Perry, 1984). In this particular case, there is much more heat available from the fuel than required by the kilning process. This excess heat can be used to produce steam which provides a credit of $4.9/tonne of 100-psig steam.

$$q_{req} = q_{CaCO_3} + q_{rxn} + q_{H_2O}$$

$$q_{CaCO_3} = Mw\,\dot{m}_{CaCO_3} \int_{311}^{1173} (Cp\,dT)_{CaCO_3}$$

$$q_{CaCO_3} = 4.1\times10^6\,\frac{Btu}{h}$$

$$q_{rxn} = Mw\,\dot{m}_{CaCO_3}\,\Delta H_{rxn}$$

$$\Delta H_{rxn} = 41{,}600\,\frac{cal}{mole\,CaCO_3}$$

$$q_{rxn} = 7.1\times10^6\,\frac{Btu}{h}$$

$$q_{H_2O} = Mw\,\dot{m}_{H_2O}\,[Cp_l(373-311) + \Delta H_{vap} + \int_{373}^{1173} (Cp_g\,dT)_{H_2O}]$$

$$q_{H_2O} = 16\times10^6\,\frac{Btu}{h}$$

$$q_{req} = 27.2\times10^6\,\frac{Btu}{h}$$

$$q_{avail} = \frac{3.3\ \ tonne fuel}{h} \times \frac{2200\ \ lbm}{tonne} \times \frac{8000\ \ Btu}{lbm} = 58.1 \times 10^6 \frac{Btu}{h}$$

$$q_{excess} = (58.1 - 27.2) \times 10^6 \frac{Btu}{h} = 30.9 \times 10^6 \frac{Btu}{h}$$

$$\dot{m}_{steam} = \frac{30.9 \times 10^6\, Btu}{h} \times \frac{lbm\ steam}{889\, Btu} = 34{,}800 \frac{lbm\ steam}{h}$$

$$Steam\ credit = \frac{34{,}800\ \ lbm}{h} \times \frac{tonne}{2200\, lbm} \times \frac{\$4.9}{tonne} \times \frac{h}{40\, tonne}$$

$$Steam\ credit = \$1.94 / tonne\ grass$$

FINAL REPORT

DEVELOPMENT OF ALTERNATIVE PRETREATMENT AND BIOMASS FRACTIONATION PROCESSES: LIME PRETREATMENT

NREL SUBCONTRACTOR REPORT

Part III
Laboratory Results for Poplar Wood

by

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Under Prime Contract DE-AC02-83CH10093

November 1996

Preface

Previously, we showed that for switchgrass, lime pretreatment is effective, economical, easy-to-apply, and environmental-friendly (Chang, *et al.*, 1996a, 1996b). Switchgrass is a low-lignin, herbaceous biomass. The purpose of this study was to determine if a high-lignin, woody biomass responds to lime pretreatment.

According to the estimates of the U. S. Department of Energy (DOE), woody biomass will amount to 48% of the available biomass resource in the U. S. in 2000, whereas agricultural residues only amounts to 24% (Stout, 1980). Furthermore, the cellulose content of woody biomass is usually higher than that of herbaceous biomass. Therefore, if woody biomass can be utilized as an energy source, it should be able to significantly meet human demand.

Unfortunately, it is well known that woody biomass has higher lignin content than herbaceous biomass and the cellulose crystallinity of woody biomass is also higher than that of herbaceous biomass. These characteristics make woody biomass a tough material for bioconversion processes. An efficient pretreatment would be the key to opening the door for woody biomass as an energy source. Now we are facing a problem, that is, does lime pretreatment work for woody biomass as it does for herbaceous biomass?

This report describes the methods and the results we obtained in the past year to explore the effectiveness of lime pretreatment for poplar wood. I would like to express my sincere appreciation to the National Renewable Energy Laboratory for their financial support and technical recommendations. I would like to thank Dr. Mark Holtzapple, Dr. Carol Holtzapple, and Dr. Richard Davison for their guidance, assistance, and encouragement.

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Summary

Lime (calcium hydroxide) was used as the pretreatment agent to enhance the enzymatic digestibility of poplar wood. Because of the high lignin content of wood, addition of oxygen to lime pretreatment was necessary to significantly enhance the enzymatic digestibility of poplar wood. The effect of lime pretreatment on digestibility at various pretreatment conditions has been studied. The recommended pretreatment conditions were: time = 6 h, temperature = 150°C, lime loading = 0.1 g/g dry biomass, water loading = 9 mL/g dry biomass, oxygen pressure = 14.8 bar absolute. Studies on the effect of particle size indicate that even coarse particles (10-20 mesh) digested well. The effect of cellulase loading for enzymatic hydrolysis has been studied and effective hydrolysis was obtained with 5 FPU/g dry biomass. High carbohydrate conversions (100% for glucan and 88% for xylan) were obtained due to the oxidative lime pretreatment. Under the recommended pretreatment and hydrolysis conditions, the glucose yield was 11 times that of untreated poplar wood, the xylose yield was 37 times, and the total sugar yield was 12 times. Using simultaneous saccharification/fermentation, the best ethanol yield was 73% of theoretical. A material balance study showed that no glucan was solublized due to the lime pretreatment whereas about 49% of xylan and 78% of lignin became solublized. About 21% of added calcium could be recovered by carbonating the wash water. To recover most (93%) of this recoverable calcium, only one wash was necessary.

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Introduction

The oil crisis first became a problem in 1973. Although many people advocate reducing energy usage, the energy consumption increases with the larger population. At current consumption rates, Krenz (1976) estimates that liquid fossil fuels will be able to meet demand for no longer than a century. Perhaps this prediction is somewhat pessimistic; however, it reveals an urgent need for human beings to seek an alternative energy source that is inexhaustible.

Bioconversion Processes

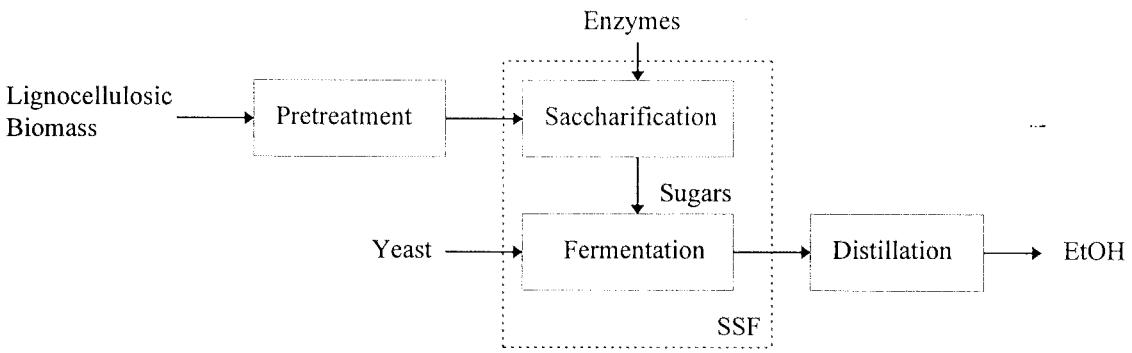
Lignocellulosic biomass is one of the most valuable alternative energy sources because it is renewable, widely available, and environmentally friendly. Using proper processes, lignocellulosic biomass can be converted to liquid fuels such as ethanol (Klyosov, 1986) or chemicals such as volatile fatty acids (Blasig *et al.*, 1992). Generally, there are two types of bioconversion processes which convert lignocellulosic biomass to alcohols (Figure 1). The traditional approach converts lignocellulosic biomass to sugars (mainly glucose and xylose) using enzymatic hydrolysis and then converts the sugars to ethanol using yeast fermentation. A modification of this approach is to merge the two stages, enzymatic hydrolysis and yeast fermentation, into one stage called simultaneous saccharification/fermentation (SSF). The alternative approach converts lignocellulosic biomass directly into volatile fatty acids (VFA) using rumen microorganisms. The VFA can be thermally converted to ketones that can be hydrogenated to mixed alcohol fuels (e.g., isopropenol, isobutanol, isopentanol, etc.).

Availability of Biomass as an Energy Source

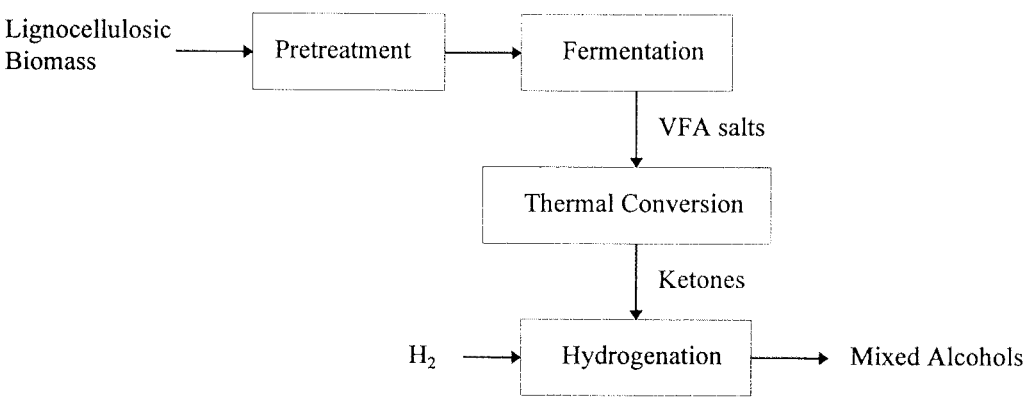
A variety of lignocellulosics have been evaluated for their availability as energy sources. Although lignocellulosic biomass is renewable and some herbaceous plants, such as forage grasses and agricultural crops, can be considered as potential energy sources due to their rapid growth, it is questionable whether there is enough arable land left to produce these "energy corps" and satisfy world food production needs. To reduce pressure on arable land, utilizing cellulosic wastes as an alternative energy source is promising because it reduces the disposal costs and because there are large quantities in agriculture and industry.

Wood residues are a significant fraction of cellulosic wastes. In the United States, in addition to the 3 million tons of wastes that are collected each year from the pulp and papermaking industries, there are about 20 million tons per year of uncollected wood processing wastes from logging, lumber, and plywood manufacturing (Horton *et al.*, 1980). Besides the wastes produced by human beings, there is also a vast quantity of forest residues created by nature. About 22% of the land on earth is covered by large forests. Unfortunately, as much as 40% of the organic material cannot be utilized with present harvesting methods and thus is left in forests to decompose by microorganisms (Wiegel, 1982). These forest residues amount to 145 million tons per year in the United States (Horton *et al.*, 1980). According to the estimates of the U. S. Department of Energy (DOE), in the year 2000, there will be 498 million dry tonne[1] /year of wood available as an alternative fuel resource in the U. S. This accounts for 48% of total available biomass resources (Stout, 1982). If these 498 million dry tonnes of wood are all to be converted to ethanol, assuming that 50% of dry wood is cellulose, there will be a tremendous theoretical ethanol yield of 141 million tonne/year. Given that the heat of combustion of ethanol is 326.7 kcal/mol (Maron *et*

[1] tonne = metric ton = 1000 kg.



(a)



(b)

Figure 1. Bioconversion processes: (a) Traditional approach; (b) Alternative approach.

al., 1974), the total energy generated by this biomass fuel annually would be 4.2×10^{18} J [2]. This only meets about 5% of the energy needs of the Unites States. Thus, national energy self-sufficiency requires a combination of many energy feedstocks (Ranney et al., 1980).

The above information provides a clear view; that is, woody biomass is a good candidate for bioconversion processes. Of many kinds of woods available in the U. S., hybrid poplar is chosen as the substrate in this research because: (1) it is a hardwood, which is usually more digestible than softwood (Baker et al., 1975); (2) it is relatively richer in cellulose than other common American woods (Timell, 1957); and (3) it is widely planted because it grows rapidly (Edlin, 1969).

Pretreatments

Although the two processes (Figure 1) have different conversion philosophies, they have one step in common: pretreatment. Without pretreatment, the susceptibility of lignocellulosic biomass to enzymatic hydrolysis or direct fermentation is constrained by structural characteristics such as cellulose crystallinity, hemicellulose acetylation, inaccessible surface area, and lignin content (Fan *et al.*, 1982; Kong *et al.*, 1992). In general, pretreatment methods are of three types: physical, chemical, and biological. Many researchers have investigated the feasibility of these pretreatment methods using a variety of lignocellulosic biomass such as forages, wood residues, crop residues, and municipal solid waste (Lin *et al.*, 1981; Chang *et al.*, 1982; Fan *et al.*, 1987; Weil *et al.*, 1994; Chang *et al.*, 1996a, 1996b). Because poplar wood is studied in this research, this section will only focus on the pretreatments that use woody biomass as their substrates, especially poplar- or aspen-related wood species[3].

Physical pretreatments can be classified as two types: mechanical and non-mechanical. Table 1 is the summary of physical pretreatment conditions and results from poplar and aspen woods. It shows that, by reducing biomass particle sizes and decreasing cellulose crystallinity, mechanical pretreatment is able to increase the biomass digestibility up to 78% using a long milling period (i.e., 2-5 h). However, such long milling times would result in high energy costs. Steam pretreatment and steam explosion are common non-mechanical pretreatment methods. Amazingly, the later increases biomass digestibility to 99%. However, applying such a high pressure (i.e., 450 psi) increases equipment costs.

Chemical pretreatments are developed to overcome the disadvantages of physical pretreatments (i.e., high energy and equipment costs), including solvent extraction, swelling, gas, acid, and alkali pretreatments. Table 2 summarizes the conditions and results of chemical pretreatment from poplar and aspen woods. Among the chemicals that have been used as pretreatment agents, alkalis seem to be the most popular agent not only because they are less expensive than other chemicals such as acids, but also because simpler processes can be applied without complicated personnel training. Sodium hydroxide and ammonia are two common alkali pretreatment agents. Although they are able to increase the biomass digestibility to a significant extent, they have several disadvantages (see Table 3). In contrast,

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[2] Theoretical ethanol yield $= 0.568 \times \frac{498 \times 10^6 \text{ tonnes dry wood}}{\text{year}} \times \frac{50 \text{ g cellulose}}{100 \text{ g dry wood}} = 141 \times 10^6 \text{ tonnes ethanol/year}$

where 0.568 is the theoretical factor for converting cellulose to ethanol.

Total energy $= \frac{326.7 \text{ kcal}}{\text{mol}} \times \frac{4.19 \times 10^3 \text{ J}}{1 \text{ kcal}} \times \frac{1 \text{ mol}}{46 \text{ g}} \times \frac{10^6 \text{ g}}{1 \text{ tonne}} \times \frac{141 \times 10^6 \text{ tonne}}{\text{year}} = 4.2 \times 10^{18} \text{ J/year}$

[3] There are two species of aspen commonly seen in the U. S.: trembling aspen (*Populus tremuloides*) and bigtooth aspen (*Populus grandidentata*). Both are also called poplar (Schoonover, 1957).

Table 1. Physical Pretreatment Conditions[a] and Results from Poplar and Aspen Woods

Pretreatment	Biomass	Temperature (°C)	Time	Other Conditions	Effect on Digestibility (%)	Reference
Mechanical						
Vibratory ball milling	Aspen	2	5 h	-----	5 to 78[b]	Pew (1957) Pew *et al.* (1962)
Vibratory ball milling	Trembling aspen	4	10, 20, 60, **120** min	-----	37 to 78[c]	Millet *et al.* (1970)
Vibratory ball milling	Trembling aspen	-----	30 min	-----	11 to 56[b]	Moore *et al.* (1972)
Non-mechanical						
Steam	Aspen poplar	140, 160, **165**, 170, 175, 180, 190, 200	0.5, 1, **2** h	-----	23.4 to 56.6[c]	Bender *et al.* (1970)
Steam	Trembling aspen	190	12, **25**, 50, 100 min	-----	75[d]	Brownell *et al.* (1987)
Steam explosion	Trembling aspen	240	20, 40, **80**, 120, 180, 240 s	-----	75[d]	Brownell *et al.* (1987)
Steam explosion	Hybrid poplar	-----	2, **5**, 20 min	Pressure = 250, **450** psi	18 to 99[d]	Grethlein *et al.* (1991)
High energy irradiation	Trembling aspen	-----	-----	Radiation dosage = 10^6, 10^7, 5×10^7, $\mathbf{10^8}$ rep.[e]	55 to 78[c]	Millet *et al.* (1970)

[a] Bold conditions correspond to the best pretreatment results.
[b] Enzymatic digestibility
[c] *in vitro* dry matter digestibility (IVDMD)
[d] Glucose yield
[e] rep. = roentgen equivalent physical

Table 2. Chemical Pretreatment Conditions[a] and Results from Poplar and Aspen Woods

Pretreatment	Biomass	Temperature (°C)	Time	Pretreatment Agent Loading	Effect on Digestibility (%)	Reference
Solvent Extraction						
MeOH/H_2O	Trembling aspen	165	1, **2.5**, 4, 7.5 h	4 g/g dry biomass	75.2[b]	Chum *et al.* (1988)
Swelling Agent						
n-Propylamine	Tulip Poplar	0	24 h	10 g/g dry biomass	60.7[c]	Kitsos *et al.* (1992)
Gas						
NH_3 (gaseous)	Trembling aspen	30	1, 2.5, 4, **8.5**, 16, 40, 73 h	155 psi at 30°C	33 to 51[d]	Millett *et al.* (1970)
SO_2	Aspen	120	2 h	30 psi at room temp.	9 to 63[e]	Baker *et al.* (1975)
Acid						
H_2SO_4	Poplar	162 to 222	3.6 to 12.7 s	0 to 1.5%	21.4 to 74.9[b]	Knappert *et al.* (1981)
H_2SO_4	Aspen	95, 120, **140**, 160	0 to 45 h	0.5 %	14 to 80[b]	Grohmann *et al.* (1985)
H_2SO_4	Hybrid Poplar	180 to 220	7 to 8 s	1%	4 to 38[b]	Grethlein *et al.* (1991)
H_2SO_4	Hybrid Poplar	170	10 min	0.73%	79[b]	Hsu *et al.* (1996)
Alkali						
NaOH	Poplar[f]	N/A[g]	N/A[g]	0 to 15 g/g dry biomass	5 to 50[d]	Wilson *et al.* (1964)
NaOH	Trembling aspen	ambient	1, **2** h	0.02, 0.04, **0.06**, 0.08, 0.1, 0.2 g/g dry biomass	35 to 55[e]	Feist *et al.* (1970)
NaOH	Trembling aspen	121	0.5, 1.0, **1.5** h	0, 2, **4**, 6%	18 to 72[h]	Huffman *et al.* (1971)
NaOH	Bigtooth aspen	ambient	2 h	0.05 g/g dry biomass	41 to 52[i]	Mellenberger *et al.* (1971)
NaOH	Trembling aspen	30	1 h	0.2 g/g dry biomass	11 to 51[j]	Moore *et al.* (1972)
NH_3 (liquid)	Trembling aspen	30	1 h	------	33 to 51[e]	Millett *et al.* (1970)
NH_3 (liquid)	Trembling aspen	30	1 h	150 psi	11 to 36[j]	Moore *et al.* (1972)
NH_3 (supercritical)	Aspen	175	20 min	0.3 g/mL (≈ 230 psi)	18.9 to 100[h]	Chou (1986)
$NH_3 + H_2O_2$	Hybrid poplar	170	90 min	NH_3 = 20 wt%; H_2O_2 = 0.28 g/g dry biomass	90[b]	Kim *et al.* (1996)

[a] Bold conditions correspond to the best pretreatment results.
[b] Glucose yield
[c] Enzymatic digestibility
[d] *in vitro* dry matter digestibility (IVDMD)
[e] *in vitro* digestibility (IVD)
[f] *Populus alba*
[g] Not available in the literature
[h] Cellulose digestion
[i] *in vivo* dry matter digestibility
[j] Reducing sugar yield

lime (calcium hydroxide) has many advantages; it is very inexpensive, is safe, and is potentially recovered by carbonating wash water (Nagwani, 1992). Unfortunately, lime has seldom been used as a pretreatment agent because most researchers usually concluded that lime was not as effective as sodium hydroxide (Abou-Raya *et al.*, 1964; Waller *et al.*, 1975; Rounds *et al.*, 1976; Wilkinson *et al.*, 1978; Owen *et al.*, 1980; Ibrahim *et al.*, 1983; Felix *et al.*, 1990) and ammonia (Oliveros *et al.*, 1993; Ibrahim *et al.*, 1983; Felix *et al.*, 1990). However, many of these comparisons employed the same pretreatment conditions, i.e., regardless of the alkali studied, equal amounts of water and alkali were used. Because calcium hydroxide is a weak alkali and poorly soluble in water, these studies put lime at a disadvantage. When modifying the pretreatment conditions to be compatible with lime, calcium hydroxide is as effective as other alkalis in enhancing lignocellulose digestibility (Owen *et al.*, 1984; Chang *et al.*, 1996b).

Overview of Research Plan

In our past studies, the lime pretreatment process has been developed for bagasse (Nagwani, 1992) and switchgrass (Chang *et al.*, 1996a, 1996b). The purpose of this research was to explore the effects of lime pretreatment on a typical woody biomass, hybrid poplar. The specific objectives were:

1. Optimize the pretreatment conditions (i.e., time, temperature, lime loading, water loading, particle size, and oxygen pressure).
2. Perform material balances to determine how much biomass is solubilized due to the lime pretreatment.
3. Determine the effect of cellulase loading on enzymatic hydrolysis.
4. Produce ethanol from pretreated biomass using simultaneous saccharification/fermentation (SSF).
5. Use carbon dioxide to recover lime as would be used in industrial processes.

Table 3. Comparison between Three Common Alkali Pretreatment Agents

Alkali	Price[a] ($/kg)	Operating Pressure	Health Hazard	Recoverability
NaOH	0.62	low	moderate	hard
NH_3	0.12	high	high	easy
$Ca(OH)_2$	0.04	low	low	moderate

[a] Peters *et al.* (1991)

Optimization of Pretreatment Conditions

Purpose

A broad survey of reaction conditions were explored to determine which lime pretreatment conditions cause the greatest increase in poplar wood digestibility. A precision estimate of the pretreatment and hydrolysis procedures was performed to check the reproducibility.

Materials and Methods

The pretreatment conditions were systematically varied to explore the effects of process variables (i.e., time, temperature, lime loading, water loading, oxygen pressure, and biomass particle size) on digestibility. Based on our past studies with bagasse (Nagwani, 1992) and switchgrass (Chang *et al.*, 1996a, 1996b), time and temperature had the largest impact on biomass digestibility. Lime loading generally had a critical value (ca. 0.1 g $Ca(OH)_2$/g dry biomass) below which the digestibility greatly declined and above which the digestibility only increased slightly. Water loading had little effect on the digestibility. Therefore, this study was conducted to hold the low-impact variables (e.g., lime loading, water loading, and particle size) constant while systematically varying the high-impact variables (e.g., time and temperature). For herbaceous biomass, the effect of oxygen pressure was not studied because high sugar yields could be achieved without adding extra oxygen in the pretreatment. Because oxygen addition will increase the capital and operating costs, the optimization of pretreatment conditions started with the non-oxidative lime pretreatment without oxygen pressure. Table 4 shows the range of conditions explored and the sequence of studies.

Table 4. The Lime Pretreatment Conditions Explored for Poplar Wood

	Time (h)	Temperature (°C)	Lime Loading (g $Ca(OH)_2$/g dry biomass)	Extra Oxygen Pressure[a] (bar absolute)	Water Loading (mL/g dry biomass)	Particle Size (Mesh)
Study 1	1 to 24	60 to 210	0.1	0	9	-40
Study 2	0.25 to 1	210 to 250	0.1	0	9	-40
Study 3	1	200	0 to 0.3	0	9	-40
Study 4	1 to 24	120 to 180	0.1	7.9	9	-40
Study 5	3 to 7.5	150	0 to 0.3	7.9	9	-40
Study 6	7.5	150	0 to 0.3	0 to 14.8	9	-40
Study 7	1 to 10	150	0.1	0 to 35.5	9	-40
Study 8	6	150	0.1	14.8	5 to 15	-40
Study 9	6	150	0.1	14.8	9	10 to -80

[a] Oxygen pressure was measured at room temperature just prior to pretreatment.

The initial lime and water loadings (i.e., 0.1 g $Ca(OH)_2$/g dry biomass and 9 mL/g dry biomass, respectively) were based on our previous study (Chang *et al.*, 1996a, 1996b). After pretreatment, the biomass was removed to an Erlenmeyer flask and acetic acid was added to neutralize the lime until the pH reached 4.8. Citrate buffer and sodium azide were added to the flasks to keep the pH constant and prevent microbial growth, respectively. Then an enzymatic hydrolysis was performed at 50°C, where 5 FPU cellulase/g dry biomass and 28.4 CBU cellobiase/g dry biomass were added. After 3 days of

hydrolysis, the reducing sugar yield of the biomass was measured using the DNS assay. The sugar content in the enzymes (ca. 4.2 mg eq. glucose/g dry biomass; see "Enzyme Loading Studies", Chang *et al.*, 1996a) was subtracted from the original sugar yields to determine the actual amounts of sugar produced from the pretreated biomass. After subtracting the enzyme sugars, the yields were multiplied by 1.015 (for the case of a lime loading of 0.1 g $Ca(OH)_2$/g dry biomass) to correct for calcium acetate inhibition and were called "corrected" reducing sugar yields. The correction factor of 1.015 was determined from the results of the "Acid Study" (Chang *et al.*, 1996a). The procedures for lime pretreatment, enzymatic hydrolysis, and DNS assay are shown in Appendices B, C, and E, respectively.

Results and Discussions

Non-oxidative Lime Pretreatment

Optimization of Pretreatment Time and Temperature

Figure 2a shows the reducing sugar yields as a function of pretreatment time (i.e., 1, 3, 6, 10, 16 and 24 h) at various temperatures (i.e., from 60 to 210°C). Lime pretreatment significantly enhanced the reducing sugar yields from poplar wood, as much as 4.6 times that of the untreated sample. A pretreatment time of more than 3 h is not necessary because it increases the reducing sugar only slightly. At high temperatures (i.e., above 150°C), the sugar yields even decreased with increasing pretreatment time. The cases of 200°C and 210°C were extreme examples where the sugar yields of the 24-h pretreated sample were even lower than that of untreated sample. This resulted from high-temperature degradation to form organic acids that not only catalyzed the degradation of cellulose and hemicellulose, but also neutralized lime. Figure 3 supports this hypothesis; it shows that the volume of acetic acid used to adjust the pH of the lime-pretreated biomass slurry to 4.8 decreased with increasing pretreatment time and temperature.

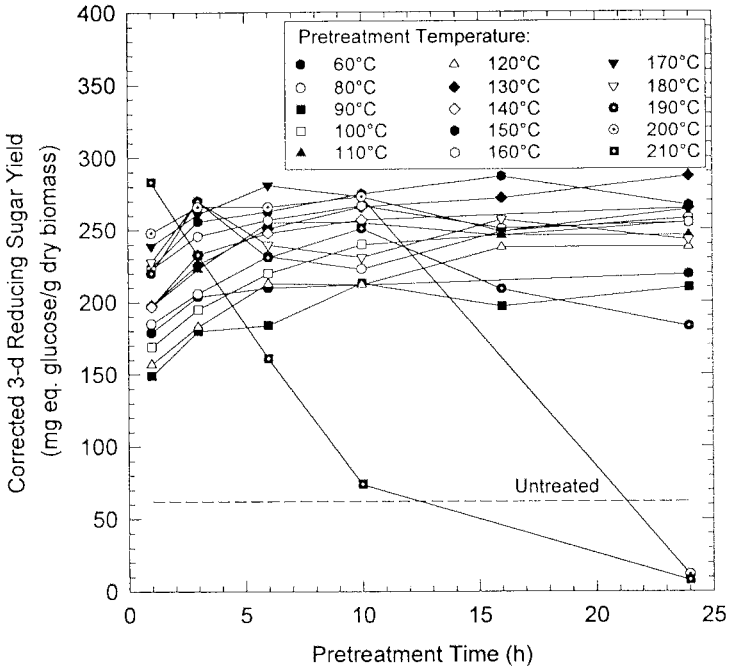
From Figure 2a, the optimal pretreatment temperature seems to lie between 150 and 200°C whereas the most effective pretreatment time seems to lie between 1 and 3 h. However, from the case of 210°C we suspected that, if the poplar wood samples were pretreated for less than 1 h, higher sugar yields might be achieved at the temperatures above 210°C. Figure 2b shows the corrected 3-d reducing sugar yields of poplar wood samples that were pretreated at 210°C to 250°C for 15, 30, and 60 min. The highest sugar yield appears when the sample was pretreated at 240°C for 30 min.

Optimization of Lime Loading

The lime loading of 0.1 g $Ca(OH)_2$/g dry biomass had been used for pretreatment. Based upon previous experience with bagasse (Nagwani, 1992) and switchgrass (Chang *et al.*, 1996a, 1996b), we expected that the reducing sugar yields would increase with lime loadings until a maximal yield was reached. After performing a 1-h pretreatment at 200°C using a range of lime loadings from 0 to 0.3 g $Ca(OH)_2$/g dry biomass, however, Fig. 4 showed that the sugar yields decreased as lime loadings increased. This might have resulted from the organic acids formed from the biomass degradation during high-temperature pretreatment (Krochta *et al.*, 1984). The organic acids actually served as pretreatment and hydrolysis reagents that were neutralized by lime. At higher lime additions there was less organic acids; thus, the "acidic pretreatment" had less effect on biomass digestibility. Therefore, at high pretreatment temperatures such as 200°C, the lime becomes a neutralization reagent rather than a pretreatment agent.

Oxidative Lime Pretreatment

Because the amount of lignin of wood is relatively high (ca. 20-35%), it is more difficult to enhance the digestibility of wood than herbaceous materials. During lime pretreatment, the lime does not function as



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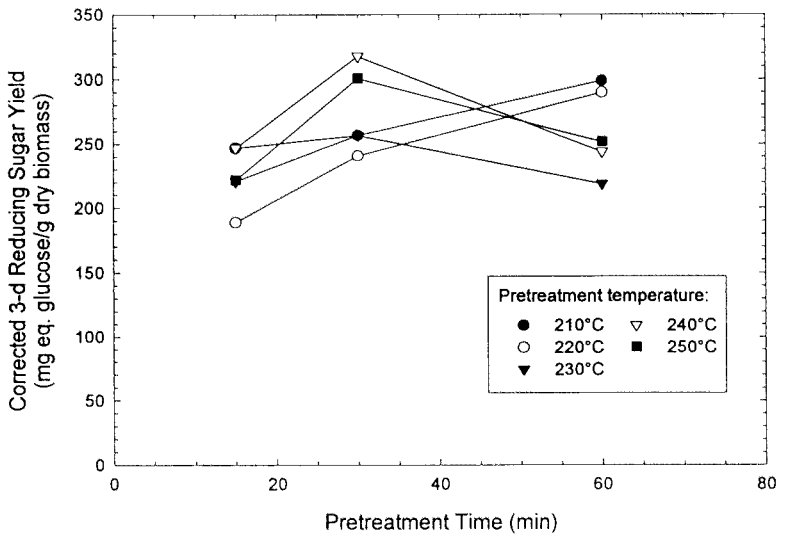


(b)

Figure 2. Optimization of pretreatment time and temperature (Non-oxidative lime pretreatment): (a) 60 to 210 °C, 1 to 24 h; (b) 210 to 250 °C, 15 to 60 min
(Pretreatment conditions: 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass)

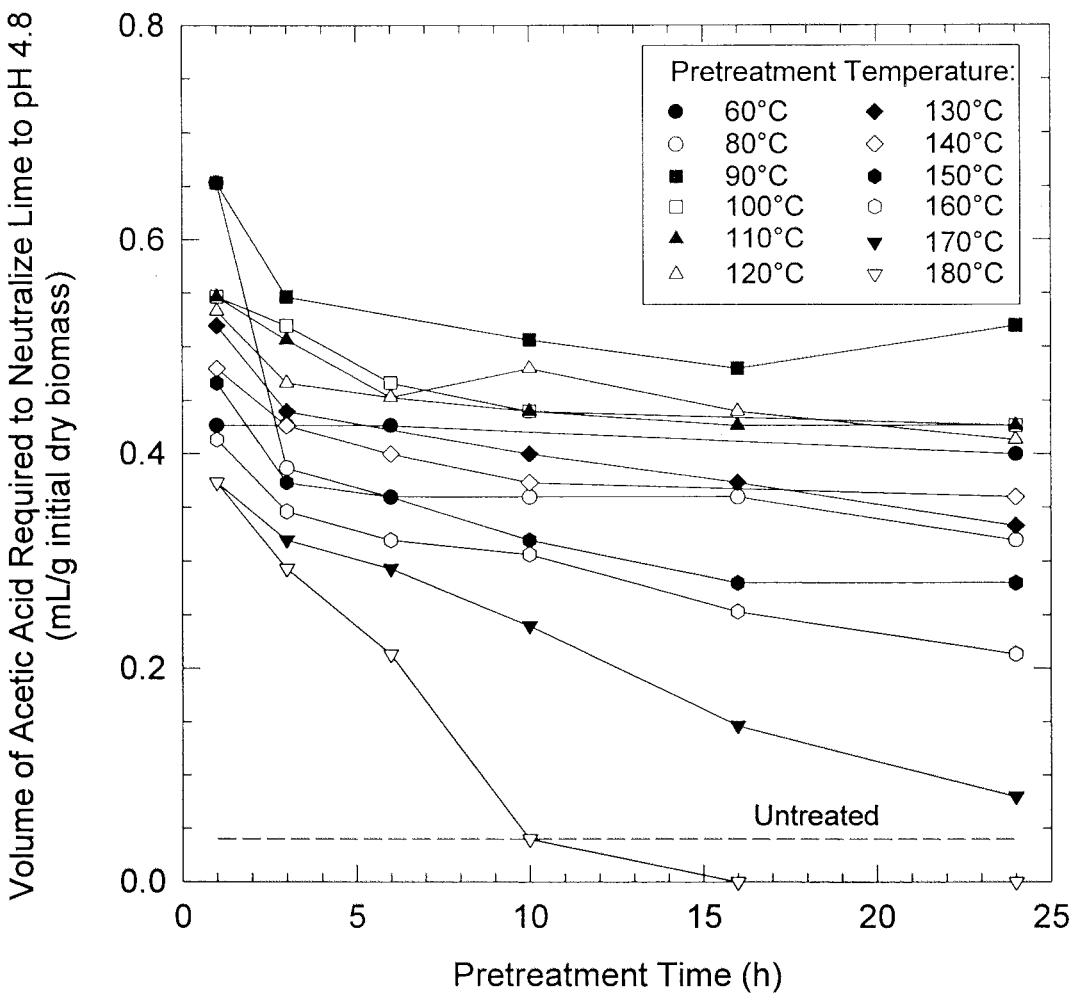


Figure 3. Volume of acetic acid required to neutralize the biomass slurry to pH 4.8 before enzymatic hydrolysis (Non-oxidative lime pretreatment)
(Pretreatment conditions: 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

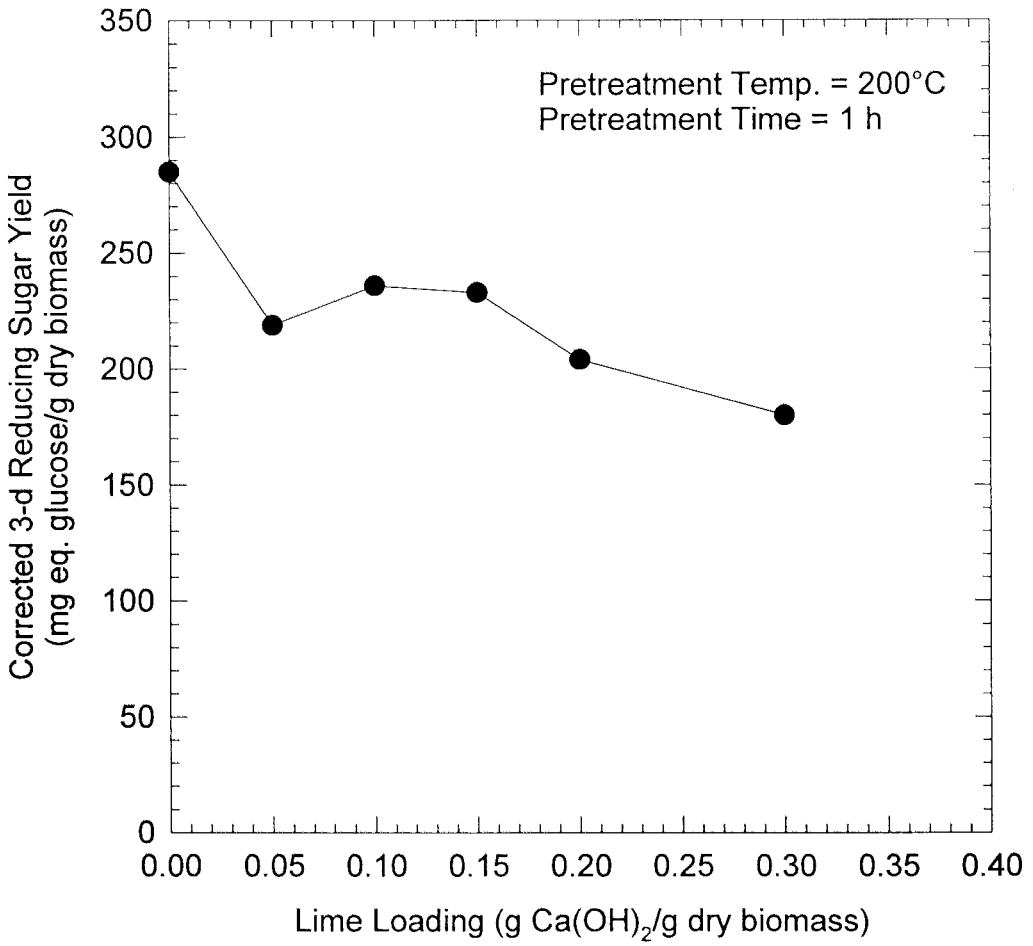


Figure 4. Optimization of lime loading (Non-oxidative lime pretreatment) (Pretreatment conditions: 1 h, 200°C, 9 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

well on poplar wood as on switchgrass. Therefore, oxidative lime pretreatments were performed, where the oxygen was used as an oxidative agent that degrades the lignin and the lime was used as a pretreatment reagent to catalyze the degradation of lignin and enhance the digestibility of the polysaccharides.

Optimization of Pretreatment Time and Temperature

After biomass, lime (0.1 g $Ca(OH)_2$/g dry biomass), and water (9 mL/g dry biomass) were added, the reactors were filled with oxygen to 7.9 bar absolute at room temperature and then placed in boiling water for 15 min. Then the reactors were placed into an oven to be heated to desired temperature. Figure 5 shows the 3-d reducing sugar yields as a function of pretreatment time (i.e., 1, 3, 6, 10, 16 and 24 h) at various temperatures (i.e., from 120 to 180°C). The lime pretreatment tremendously enhanced the reducing sugar yields of poplar wood, as much as eight times that of the untreated sample. The highest reducing sugar yield (i.e., 513 mg eq. glucose/g dry biomass) occurred at 140°C and 16 h. However, such a long reaction time is not economical from an industrial viewpoint. Therefore, 150°C and 6 h appeared to be the optimal pretreatment temperature and time, respectively.

Addition of oxygen in lime pretreatment enhances the enzymatic digestibility of poplar wood significantly. The corrected 3-d reducing sugar yield of the sample oxidatively pretreated at 150°C and 6 h was 469 mg eq. glucose/g dry biomass, which was about 1.8 times that of the sample pretreated at the same conditions except no extra oxygen was added (i.e., 263 mg eq. glucose/g dry biomass). It indicates that, by adding oxygen as an oxidative agent, lime functions as an alkaline reagent more efficiently, especially when a high-lignin biomass, such as wood, is used as the substrate.

Optimization of Lime Loading and Pretreatment Time

A lime loading of 0.1 g $Ca(OH)_2$/g dry biomass had been applied in the experiments so far. Although Fig. 5 shows that the optimal conditions occur at 150°C and 6 h, it was suspected that shorter reaction times might be sufficient with higher lime loadings, or less lime loadings might be enough for longer reaction times. Therefore, the optimization of lime loading was explored at 150°C for the pretreatment times close to 6 h (i.e., 3, 4.5, 6, and 7.5 h), where the lime loading varied from 0 to 0.3 g $Ca(OH)_2$/g dry biomass.

Figure 6 shows that the highest sugar yields occurred when the pretreatment time of 7.5 h was applied. However, as the pretreatment time increased, the reducing sugar yields depended less on lime loadings. For the 7.5-h pretreatment, the reducing sugar yields increased only slightly for the lime loading above 0.1 g $Ca(OH)_2$/g dry biomass, whereas for the 3-h pretreatment, the reducing sugar yield increases 59% (i.e., from 319 to 507 mg eq. glucose/g dry biomass) when the lime loading increases from 0.1 to 0.3 g $Ca(OH)_2$/g dry biomass. Fig. 6 also shows that at high lime loadings (i.e., 0.3 g $Ca(OH)_2$/g dry biomass), pretreatment time has little effect on the sugar yields. Therefore, the best conditions should be the combination of long pretreatment time (such as 7.5 h) and low lime loading (such as 0.1 g $Ca(OH)_2$/g dry biomass), or the combination of short pretreatment time (such as 3 h) and high lime loading (such as 0.3 g $Ca(OH)_2$/g dry biomass); however, a 200% increase of lime consumption may not be justified for a 59% increase of sugar yield.

Optimization of Lime Loading and Oxygen Pressure

It was expected that the reducing sugar yields would increase as the oxygen pressure increased due to the further removal of lignin. It was also suspected that less lime loading might be sufficient at higher oxygen pressure. Therefore, oxidative lime pretreatments were performed at the following conditions:

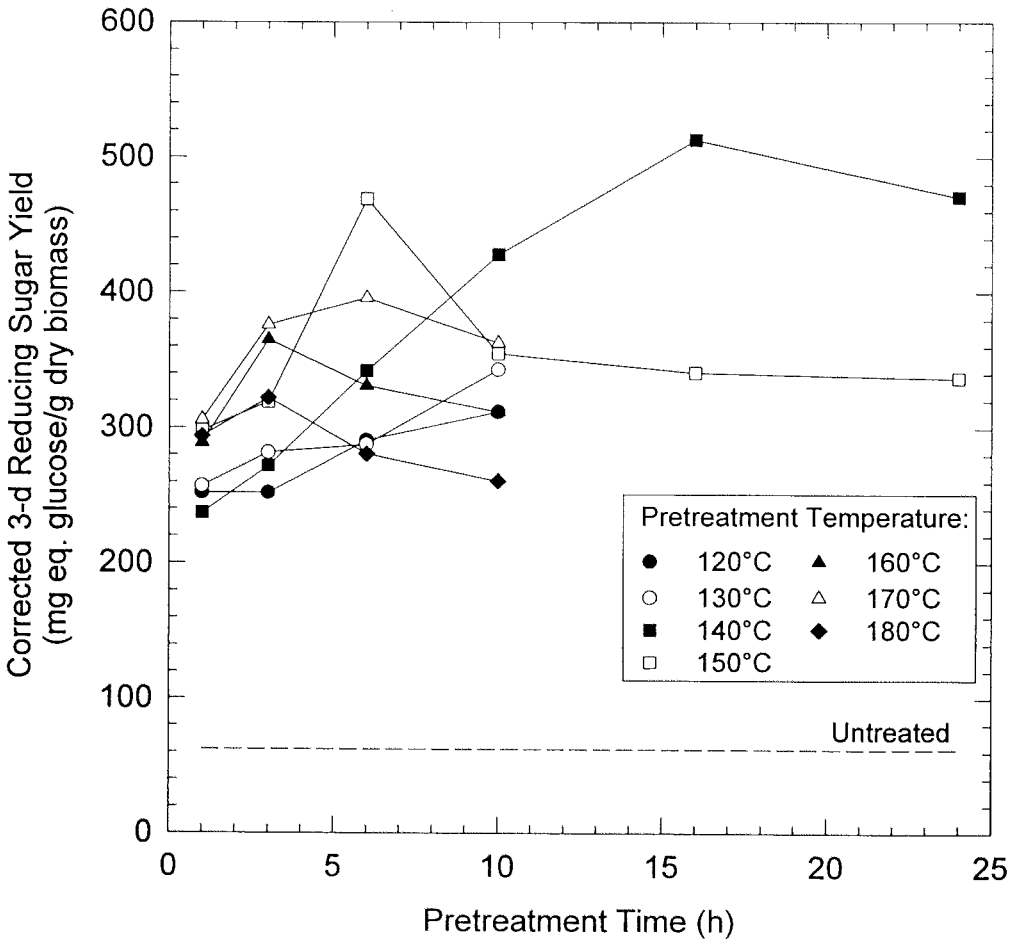


Figure 5. Optimization of pretreatment time and temperature (Oxidative lime pretreatment)
(Pretreatment conditions: 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 7.9 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass)

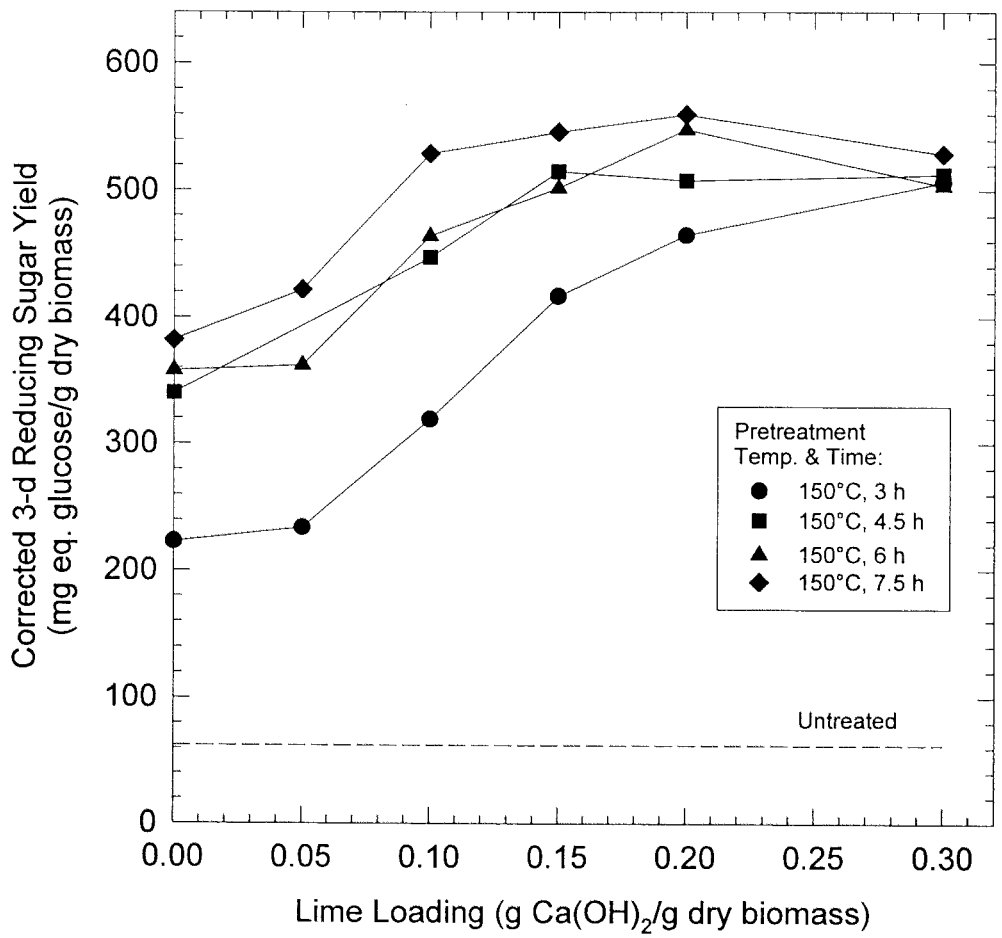


Figure 6. Optimization of pretreatment time and lime loading (Oxidative lime pretreatment) (Pretreatment conditions: 150°C, 9 mL water/g dry biomass, oxygen pressure = 7.9 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

temperature = 150°C, time = 7.5 h, oxygen pressure = 0 to 14.8 bar absolute, lime loading = 0 to 0.3 g $Ca(OH)_2$/g dry biomass, water loading = 9 mL/g dry biomass.

Figure 7 shows that, at both oxygen pressures of 7.9 and 14.8 bar absolute, the reducing sugar yields only increase slightly for the lime loadings above 0.1 g $Ca(OH)_2$/g dry biomass. Thus the optimal lime loading can be more surely determined as 0.1 g $Ca(OH)_2$/g dry biomass. At this lime loading, when compared with the reducing sugar yield of untreated poplar wood, the non-oxidative lime pretreatment increased the reducing sugar yield by 197% (i.e., from 62 to 184 mg eq. glucose/g dry biomass), the oxidative lime pretreatment at 7.9 bar absolute of oxygen pressure increased reducing sugar yields by 753% (i.e., from 62 to 529 mg eq. glucose/g dry biomass), and the oxidative lime pretreatment at 14.8 bar absolute of oxygen pressure increased reducing sugar yields by 903% (i.e., from 62 to 622 mg eq. glucose/g dry biomass). These data further illustrate the importance of oxygen addition in lime pretreatment.

Optimization of Oxygen Pressure and Pretreatment Time

Fig. 7 shows that, at the lime loading of 0.1 g $Ca(OH)_2$/g dry biomass, the reducing sugar yield increases approximately 18% as the oxygen pressure increases from 7.9 to 14.8 bar absolute. Thus, it was logical to explore the effects of higher oxygen pressures on the enzymatic digestibility of biomass. Furthermore, shorter pretreatment times might be sufficient at higher oxygen pressures. Therefore, the oxidative lime pretreatments were performed with various oxygen pressures (i.e., from 0.2[4] to 34.5 bar absolute) and different pretreatment times (i.e., 1, 3, 6, and 10 h). Other parameters were: temperature = 150°C, lime loading = 0.1 g $Ca(OH)_2$/g dry biomass, and water loading = 9 mL/g dry biomass.

Figure 8a shows that the highest reducing sugar yields occurred at 14.8 bar absolute of oxygen pressure for long pretreatment times (i.e., 6 and 10 h). Figure 8b indicates that the pretreatment times above 6 h had little effect on the reducing sugar yields, especially for oxygen pressure higher than 14.8 bar absolute. Therefore, 14.8 bar absolute is selected as the optimal oxygen pressure and 6 h is selected as the optimal pretreatment time.

Optimization of Water Loading

Fig. 9 shows the effect of water loadings on reducing sugar yields. The pretreatment conditions were: temperature = 150°C, time = 6 h, lime loading = 0.1 g $Ca(OH)_2$/g dry biomass, oxygen pressure = 14.8 bar absolute, and water loading = 5 to 15 mL/g dry biomass. Although water loadings as low as 7 mL/g dry biomass are effective, there is little economic incentive to reduce the water loadings to a bare minimum. Furthermore, the highest reducing sugar yield (i.e., 599 mg eq. glucose/g dry biomass) occurs at the water loading of 9 mL/g dry biomass. Therefore, a water loading of 9 mL/g dry biomass can be used.

Optimization of Particle Size

Fig. 10 shows the effect of biomass particle size on the digestibility. Six different particle sizes were studied (i.e., 10-20 mesh, 20-30 mesh, 30-40 mesh, 40-50 mesh, 50-80 mesh, 80 mesh and finer). The pretreatment conditions were: temperature = 150°C, time = 6 h, lime loading = 0.1 g $Ca(OH)_2$/g dry biomass, oxygen pressure = 14.8 bar absolute, and water loading = 9 mL/g dry biomass. Grinding to less than 10 mesh is sufficient for optimal pretreatment. Even though it was not necessary to grind biomass below 40 mesh, subsequent experiments were performed using a particle size of -40 mesh because there

[4] This corresponds to the non-oxidative lime pretreatment. In the non-oxidative lime pretreatment, no extra oxygen was added. Therefore, the oxygen pressure at room temperature was: 1.013 bar abs × 21% = 0.213 bar abs.

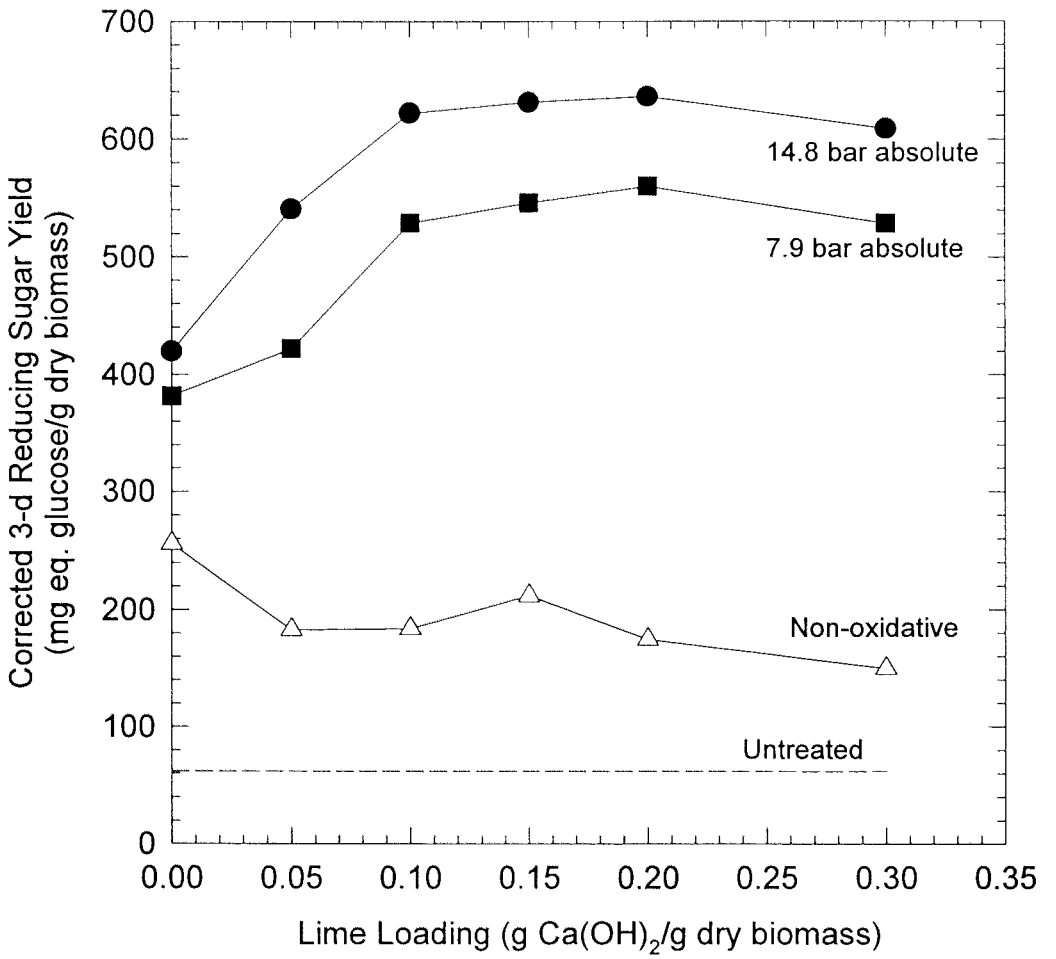
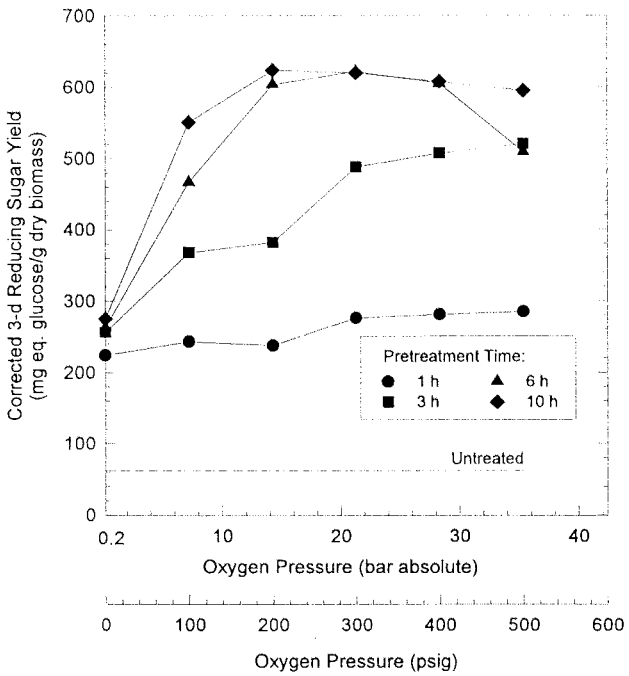
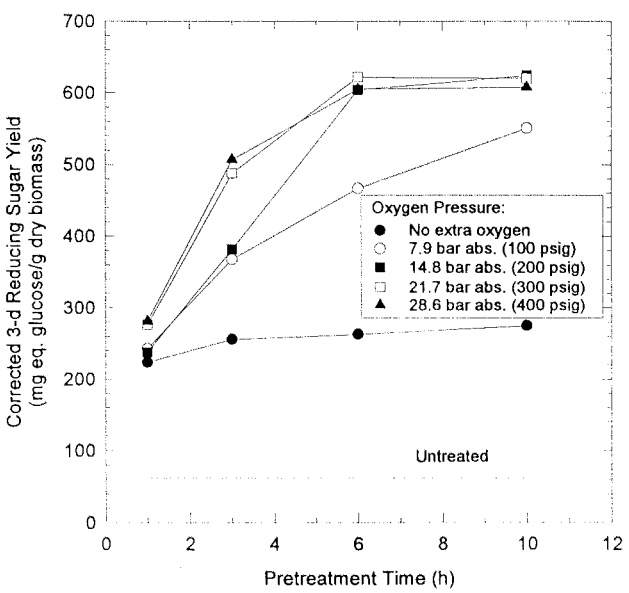


Figure 7. Optimization of oxygen pressure and lime loading (Oxidative lime pretreatment)
(Pretreatment conditions: 150°C, 7.5 h, 9 mL water/g dry biomass.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)



(a)



(b)

Figure 8. Optimization of oxygen pressure and pretreatment time (Oxidative lime pretreatment) (Pretreatment conditions: 150°C, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

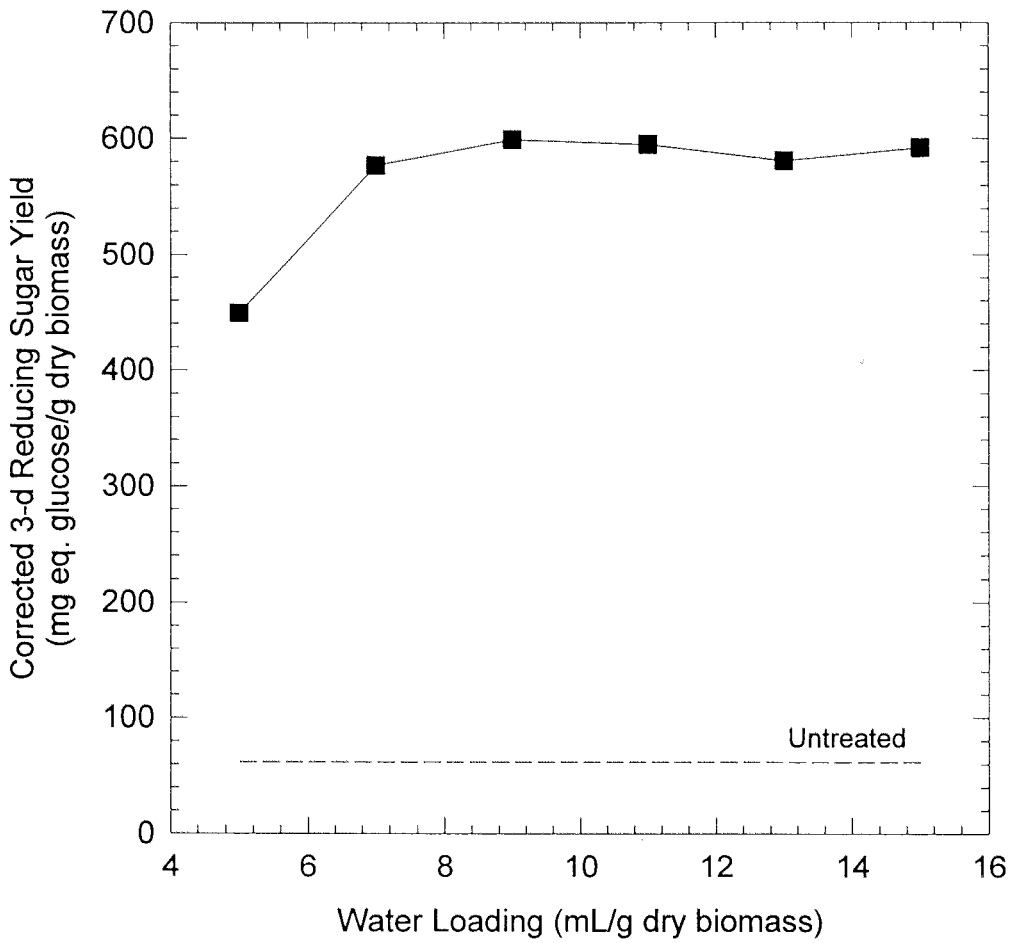


Figure 9. Optimization of water loading (Oxidative lime pretreatment)
(Pretreatment conditions: 6 h, 150°C, 0.1 g $Ca(OH)_2$/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

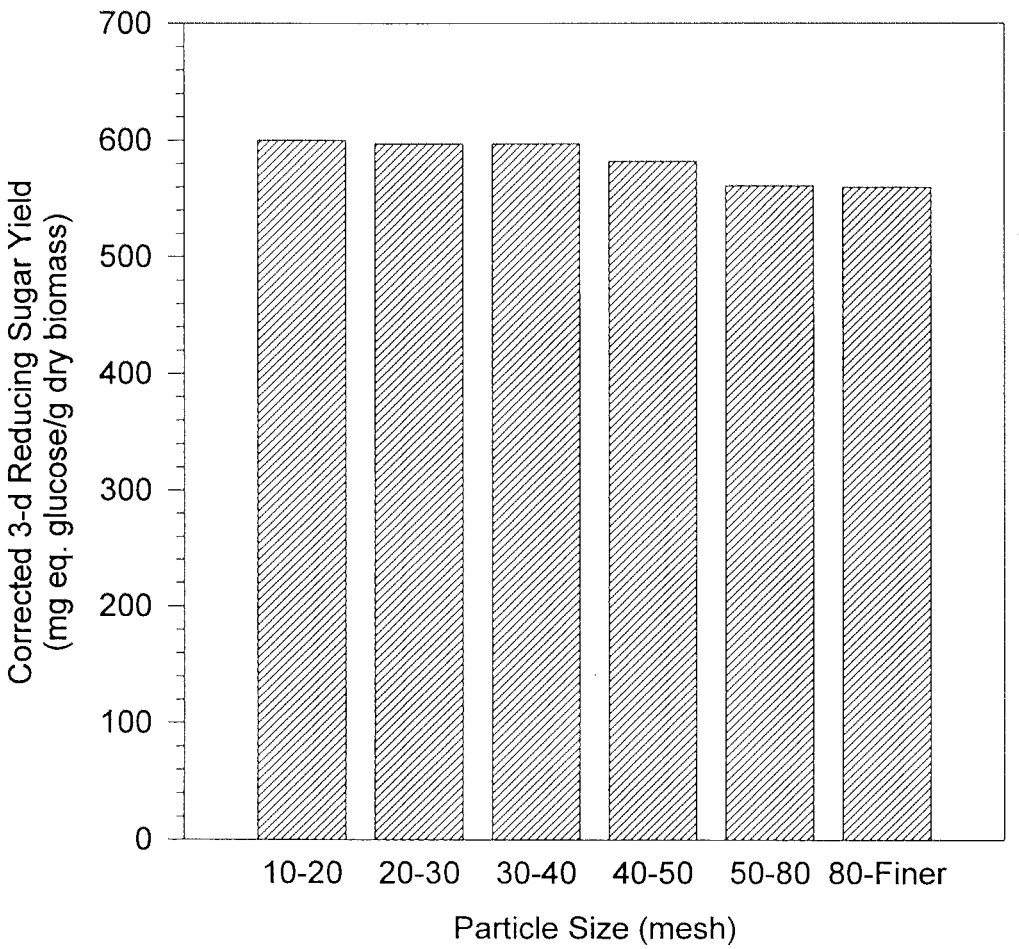


Figure 10. Particle size study (Oxidative lime pretreatment)
(Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

was a large quantity of this material available. Also, it is of a more uniform particle size which reduces variability between experiments.

Method Reproducibility

Internal and external precision tests of the oxidative lime pretreatment and enzymatic hydrolysis procedures were performed at the following conditions: pretreatment temperature = 150°C, pretreatment time = 6 h, lime loading = 0.1 g $Ca(OH)_2$/g dry biomass, water loading = 9 mL/g dry biomass, oxygen pressure = 14.8 bar absolute, biomass particle size = -40 mesh, hydrolysis pH = 9, hydrolysis temperature = 50°C, hydrolysis time = 3 d, cellulase loading = 5 FPU/g dry biomass, cellobiase loading = 28.4 CBU/g dry biomass. Internal precision was determined by preparing the reactors and pretreating simultaneously and then hydrolyzing simultaneously. External precision tests were accomplished by preparing the reactors and pretreating separately and then hydrolyzing separately. Table 5 shows the sample sizes, mean sugar yields, standard deviations, coefficients of variation. Coefficients of variation were calculated using the following formula:

$$\text{Coefficient of Variation} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100 \quad (1)$$

Table 5. Method Reproducibility of Oxidative Lime Pretreatment and Enzymatic Hydrolysis

Precision Mode	Sample Size	Mean[a]	Standard Deviation[a]	Coefficient of Variation (%)
Internal	5	570	10.9	1.9
External	6	576	19.2	3.3

[a] The units is mg eq. glucose/g dry biomass.

Conclusions

Addition of oxygen in lime pretreatment enhances tremendously the enzymatic digestibility of poplar wood. Table 6 summarizes the recommended pretreatment conditions for poplar wood. The average corrected 3-d reducing sugar yield of the samples pretreated in these conditions is 602 mg eq. glucose/g dry biomass.

Table 6. Recommended Conditions of Oxidative Lime Pretreatment for Poplar wood

Variables	Recommended Values
Time	6 h
Temperature	150°C
Lime Loading	0.1 g $Ca(OH)_2$/g dry biomass
Oxygen Pressure	14.8 bar absolute (at room temperature) 21.0 bar absolute (at 150°C)[a]
Water Loading	9 mL/g dry biomass
Particle Size	10 mesh and finer

[a] At actual reaction conditions (i.e., 150°C), the oxygen pressure is calculated as follows:
$P_{O_2 \text{ at } 150°C}$ = 14.8 bar absolute × (150 + 273.15) K/ (25 + 273.15) K = 21.0 bar absolute

Material Balances

Purpose

Material balances were performed to determine how much biomass is solubilized by the lime pretreatment.

Materials and Methods

To remove any solubles, untreated and pretreated poplar wood (pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute) were repeatedly washed with fresh distilled water until the decanted water became colorless (see Appendix D for the detailed washing procedure). The total weight (105°C dry weight) of the sample was measured before and after pretreatment and wash. The compositions (i.e., glucan, xylan, Klason lignin, acid-soluble lignin, and ash) of raw, washed-only, and pretreated-and-washed poplar wood were determined according to the NREL standard procedures No. 002 to 005. The total lignin was determined as the summation of Klason lignin and acid-soluble lignin. The crude protein content of the samples was determined by the Forage Testing Laboratory of Texas A&M University using a modified micro-Kjeldahl procedure which used sulfuric acid-hydrogen peroxide with lithium sulfate and selenium catalysts. Total nitrogen as ammonium was determined colormetrically using a Technicon Autoanalyzer II continuous segmented flow system. The nitrogen value was then multiplied by 6.25 to estimate crude protein. The mineral contents were also determined by the Forage Testing Laboratory of Texas A&M University using a nitric digest method.

Results and Discussions

Tables 7 and 8 show the compositions of raw, washed-only, and pretreated-and-washed poplar wood. Table 9 summarizes the loss percentages of each component before and after pretreatment. All the components become more water-soluble due to the oxidative lime pretreatment, except ash and glucan. As shown in Fig. 11a, fairly large quantities of lignin, xylan, crude protein, and other components (e.g., extractives) were removed by the oxidative lime pretreatment, whereas glucan was hardly removed; hence, glucan is much more stable than other components and very resistant to the oxidative lime pretreatment. About 78% of lignin removed by the oxidative lime pretreatment may contribute to the increase of the biomass digestibility. Fig. 11b shows that the compositions of raw and washed-only poplar wood are essentially similar, whereas the pretreated-and-washed biomass is enriched in cellulose (i.e., 76%).

Similar to the case of switchgrass (Chang *et al.*, 1996a, 1996b), the amount of water-insoluble ash increased after the lime pretreatment. It was suspected that it might result from the calcium which attached to the biomass during the lime pretreatment. Table 10 and Figure 12 prove the hypothesis. The calcium content of poplar wood increased substantially after the lime pretreatment (i.e., from 0.31% to 1.23%), causing the total mineral content increased from 0.78% to 1.29%. About 76% of sulfur and 84% of magnesium were removed after the lime pretreatment, whereas phosphorus and potassium were fairly soluble even before the lime pretreatment. Other minerals (i.e., boron, iron, manganese, sodium, copper, zinc and aluminum) constitute only a minor part of ash.

Table 11 summarizes the results of material balances from various pretreatments of poplar and aspen woods. Because most literature does not show the solubility of each component of raw biomass (i.e., the material balances between raw and washed-only biomass was not found), it is difficult to compare the weight loss caused by the pretreatment alone. Besides, little literature pays attention on the ash and protein contents. Therefore, only the material balances between raw and pretreated-and-washed biomass are discussed in this study for major components (i.e., lignin, glucan, and xylan). The dilute acid pretreatments have relatively high total solid recoveries (i.e., 68-77%) whereas the steam pretreatment has a low recovery of 47%. The total solid recovery of the lime pretreatment is moderate (i.e., 57%). In terms of lignin removal, organosolv, ammonia-hydrogen peroxide percolation, and lime pretreatments can remove more than 80% of lignin, whereas the dilute acid pretreatment hardly removes lignin (i.e., 0-5%). As to carbohydrate recovery, the lime pretreatment has the highest glucan recovery (i.e., 99.9%), which results in the highest glucan content in lime-treated poplar wood (i.e., 76%), and a moderate xylan recovery (i.e., 46%). The steam pretreatment removes almost all xylan (i.e., 98%) and a significant amount of glucan (i.e., 19%). The dilute acid pretreatment has the highest xylan recovery (i.e., 68%) among these pretreatments.

Because the enzymatic digestibility of lignocellulosic biomass is related to the presence of lignin and the acetylation of hemicellulose (Schurz, 1977; Kong *et al.*, 1992), these two characteristics of treated biomass are determined by the delignification and hemicellulose removal of a pretreatment. As shown in Table 11, organosolv, ammonia-hydrogen peroxide percolation, and lime pretreatments remove similar amounts of lignin (83.4% vs. 80% vs. 82.1%, respectively) and xylan (56.1% vs. 50% vs. 54.1%, respectively), and they increase the enzymatic digestibility of aspen/poplar wood to similar extents. The 100-h glucose yield of organosolv-treated aspen wood is 75.2% (cellulase loading unknown) (Chum *et al.*, 1988), whereas the 72-h glucose yield of NH_3-H_2O_2-treated poplar wood is 90% at a cellulase loading of 60 FPU/g glucan (Kim *et al.*, 1996). The 72-h glucose yield of lime-treated poplar wood is 77.3% at a cellulase loading of 5 FPU/g dry biomass and 88% at a cellulase loading of 25 FPU/g dry biomass (equivalent to 57.6 FPU/g glucan) (see "Enzyme Loading Studies" and "Hydrolysis Profiles").

Table 7. Material Balances between Raw and Washed-Only Poplar Wood

Components	Raw Sample		Washed Sample		Loss	
	Weight[a] (g)	Weight Percentage	Weight[a] (g)	Weight Percentage	Weight[a] (g)	Weight Percentage[b]
Total	45.0279	100.00%	42.8375	100.00%	2.1904	4.86%
Ash	0.5719	1.27%	0.3855	0.90%	0.1863	32.58%
Lignin[c]	12.5673	27.91%	11.9902	27.99%	0.5771	4.59%
Xylan	6.7677	15.03%	6.4085	14.96%	0.3592	5.31%
Glucan	19.5421	43.40%	19.1655	44.74%	0.3766	1.93%
Crude Protein	0.7655	1.70%	0.5954	1.39%	0.1700	22.21%
Others	4.8135	10.69%	4.2923	10.02%	0.5212	10.83%

[a] 105°C dry weight
[b] Based on the initial weight of each component before washing
[c] Lignin = Klason lignin + acid-soluble lignin

Table 8. Material Balances between Raw and Pretreated[a]-&-Washed Poplar Wood

Components	Raw Sample		Pretreated-&-Washed Sample		Loss	
	Weight[b] (g)	Weight Percentage	Weight[b] (g)	Weight Percentage	Weight[b] (g)	Weight Percentage[c]
Total	39.1929	100.00%	22.4543	100.00%	16.7386	42.71%
Ash	0.4977	1.27%	0.4311	1.92%	0.0666	13.39%
Lignin[d]	10.9387	27.91%	1.9558	8.71%	8.9830	82.12%
Xylan	5.8907	15.03%	2.7057	12.05%	3.1849	54.07%
Glucan	17.0097	43.40%	16.9934	75.68%	0.0163	0.10%
Crude Protein	0.6663	1.70%	0.1055	0.47%	0.5607	84.16%
Others	4.1897	10.69%	0.2627	1.17%	3.9270	93.73%

[a] Pretreatment conditions: temperature = 150°C, time = 6 h, lime loading = 0.1 g $Ca(OH)_2$/g dry biomass, water loading = 9 mL/g dry biomass, oxygen pressure = 14.8 bar absolute, and particle size = -40 mesh
[b] 105°C dry weight
[c] Based on the initial weight of each component before pretreatment and washing
[d] Lignin = Klason lignin + acid-soluble lignin

Table 9. Summary of Water-Solubility of Poplar Wood Components Before and After Lime Pretreatment[a]

Components	Raw Composition (g component/g total)	Weight Loss Percentage[b]		Amount Removed by Lime Pretreatment[b]
		Washed-Only	Pretreated-&-Washed	
Total	------	4.86%	42.71%	37.85%
Ash	0.06	32.58%	13.39%	-19.19%
Lignin	0.22	4.59%	82.12%	77.53%
Xylan	0.22	5.31%	54.07%	48.76%
Glucan	0.38	1.93%	0.10%	-1.83%
Crude Protein	0.08	22.21%	84.16%	61.95%
Others	0.04	10.83%	93.73%	82.90%

[a] Pretreatment conditions: temperature = 150°C, time = 6 h, lime loading = 0.1 g $Ca(OH)_2$/g dry biomass, water loading = 9 mL/g dry biomass, oxygen pressure = 14.8 bar absolute, and particle size = -40 mesh
[b] Weight percentage based on the initial weight of each component

Table 10. Mineral Content[a] of Poplar Wood

Component	Raw	Washed-Only	Pretreated-and-Washed
S	0.0281%	0.0237%	0.0019%
B	0.0020%	0.0016%	0.0007%
Mo	≈ 0	≈ 0	≈ 0
Ca	0.3085%	0.3152%	1.2272%
P	0.0392%	0.0087%	≈ 0
Fe	0.0055%	0.0045%	0.0160%
Mg	0.0876%	0.0785%	0.0018%
Mn	0.0009%	0.0009%	0.0002%
Na	0.0164%	0.0221%	0.0209%
Cu	0.0005%	0.0011%	0.0052%
Zn	0.0002%	0.0020%	0.0026%
K	0.2936%	0.0238%	≈ 0
Al	0.0004%	0.0034%	0.0097%
Total Minerals	0.7827%	0.4856%	1.2862%

[a] Based on 105°C dry weight.

Table 11. Summary of Material Balances from Various Pretreatments of Poplar and Aspen Woods

Pretreatment	Biomass	Biomass Input	Total Solid Recovered	Total Weight Loss	Compositions (%)									Reference
					Lignin			Glucan			Xylan			
					Raw[a]	Pret.[b]	Loss[c]	Raw[a]	Pret.[b]	Loss[c]	Raw[a]	Pret.[b]	Loss[c]	
Steam[d]	Trembling aspen	100 g	46.9 g	43.1 g	21.79	30.3	20.9	48.08	68.3	19.2	19.59	0.64	98.3	Schwald *et al.* (1989)
Organosolv[e]	Trembling aspen	100 g	58.1 g	41.9 g	18.4	5.2	83.4	44.4	70.3	8.0	18.4	13.9	56.1	Chum *et al.* (1988)
Dilute Acid[f]	Poplar	100 g	77.2 g	22.8 g	N/A[k]	N/A[k]	N/A[k]	45.7	57.9	2.2	17.3	15.1	32.4	Knappert *et al.* (1981)
Dilute Acid[g]	Aspen	100 g	68-74 g	26-32 g	18[l]	26-29[l]	0-5[l]	50	60-66	N/A[k]	18	0-3	N/A[k]	Grohmann *et al.* (1986)
Dilute Acid[h]	Hybrid poplar	100 g	N/A[k]	N/A[k]	27.9	33.9	N/A[k]	43.7	65.1	N/A[k]	16.0	2.6	N/A[k]	Hsu *et al.* (1996)
NH_3-H_2O_2 Percolation[i]	Hybrid poplar	100 g	N/A[k]	N/A[k]	N/A[k]	N/A[k]	80	N/A[k]	N/A[k]	2	N/A[k]	N/A[k]	50	Kim *et al.* (1996)
Lime[j]	Hybrid poplar	100 g	57.29 g	42.71 g	27.91	8.71	82.12	43.40	75.68	0.10	15.03	12.05	54.07	This work

[a] Untreated biomass. Compositions are determined based on the dry weight of untreated biomass.

[b] Pretreated-and-washed biomass. Compositions are determined based on the dry weight of pretreated-and-washed biomass.

[c] Based on the initial dry weight of each component before pretreatment and washing.

[d] Pretreatment conditions: 210°C, 100 s, $[SO_2]$ = 1.6% (as a catalyst)

[e] Pretreatment conditions: 165°C, 2.5 h, MeOH:H_2O = 70:30

[f] Pretreatment conditions: 200°C, 5.8 s, $[H_2SO_4]$ = 0.41%

[g] Pretreatment conditions: 140-160°C, 0-100 min, $[H_2SO_4]$ = 0.45-2.5%

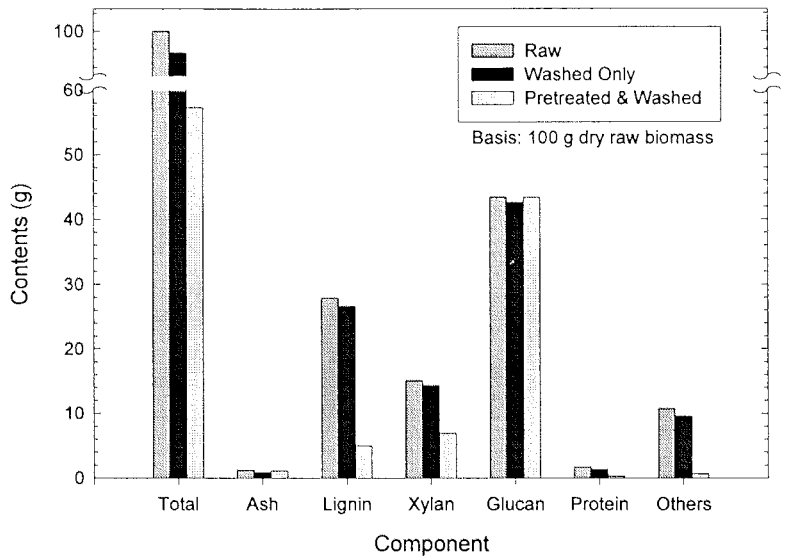
[h] Pretreatment conditions: 170°C, 10 min, $[H_2SO_4]$ = 0.73%

[i] Pretreatment conditions: 170°C, 90 min, $[NH_3]$ = 20 wt%, 0.28 g H_2O_2/g dry biomass

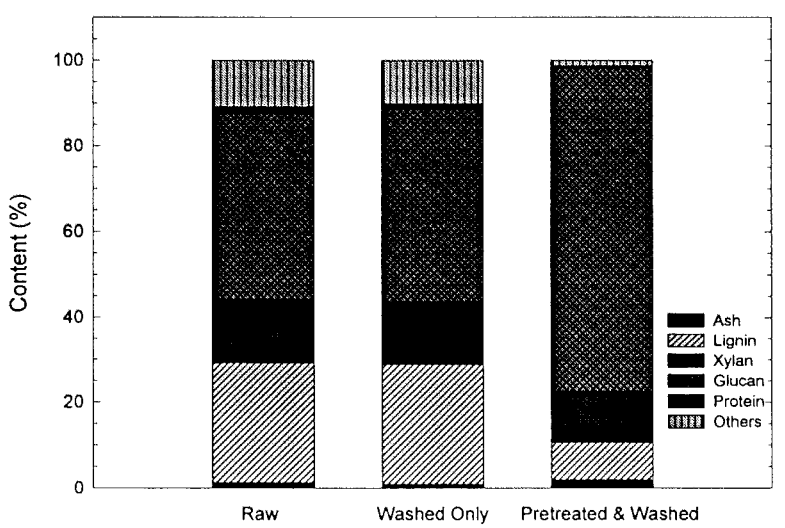
[j] Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute

[k] Not available in the literature.

[l] Klason lignin only.



(a)



(b)

Figure 11. Composition of raw, washed-only, and pretreated-and-washed poplar wood: (a) relative composition; (b) absolute composition.
(Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute)

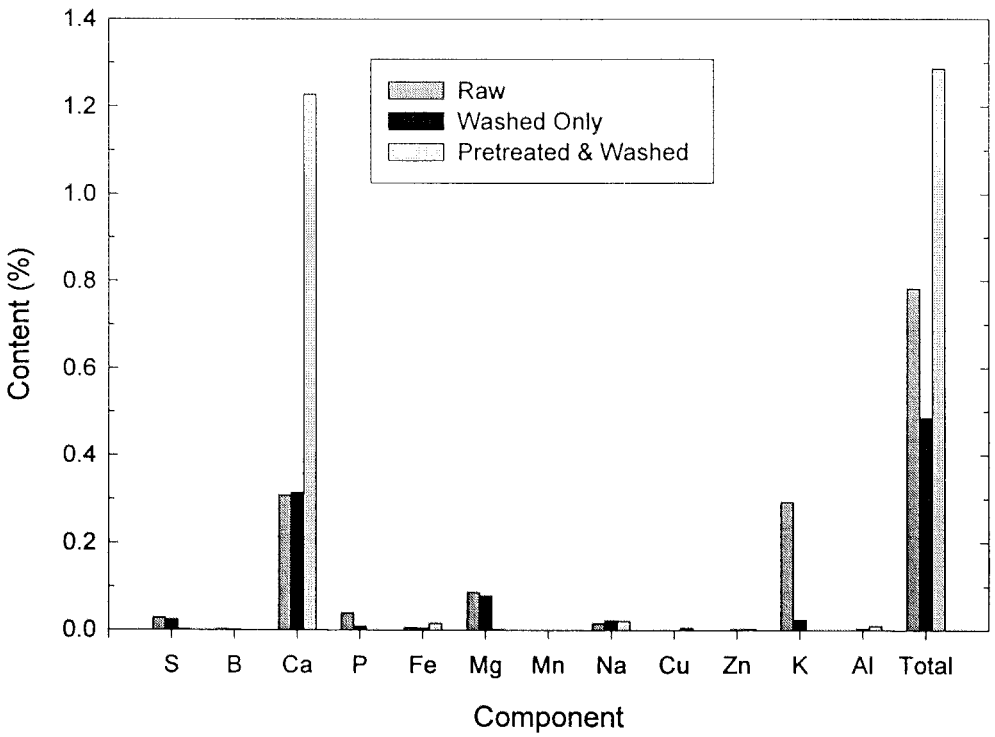


Figure 12. Material balances on minerals of poplar wood.

Enzyme Loading Studies

Purpose

A cellulase loading of 5 FPU/g dry biomass had been used in our previous studies. This study of cellulase loading is to determine if there are yield benefits from loadings higher than 5 FPU/g dry biomass or if the cellulase loadings less than 5 FPU/g dry biomass are acceptable. The sugar content of the enzymes were also to be explored.

Materials and Methods

Filter Paper Assay

To verify the activity of the cellulase received from NREL, a filter paper assay was performed according to the NREL standard procedure No. 006. The filter paper activity of the cellulase (Cytolase CL enzyme, Lot No. 17-92262-09) was 89.7 FPU/mL. The activity reported by the manufacturer (Environmental BioTechnologies, Inc.) was 95.3 FPU/mL.

Determination of Sugar Content of the Enzymes

The enzymes were incubated at 50°C, pH 4.8 for 3 days, as explained in Appendix C, "General Enzymatic Hydrolysis Procedure," except that no biomass was involved. The resulting reducing sugars were determined by the DNS assay and the resulting glucose and xylose were determined using HPLC (Appendix E). The experiments were performed for two combinations of the enzymes: 0.852 mL cellobiase, and 0.852 mL cellobiase + 6.271 mL cellulase. The amount of cellobiase (i.e., 0.852 mL) was chosen because it was our standard cellobiase loading in the enzymatic hydrolysis of 7.5 g dry biomass[5]. The amount of cellulase (i.e., 6.271 mL) was chosen because it corresponded to the highest cellulase loading (i.e., 75 FPU/g dry biomass) that we explored in this cellulase loading studies[6]. The sugar content of other enzyme combinations were approximated using an interpolation method.

Study of Cellulase Loading

Approximately 90 g (105°C dry weight) of poplar wood was pretreated under the recommended conditions (i.e., time = 6 h, temperature = 150°C, lime loading = 0.1 g $Ca(OH)_2$/g dry biomass, water loading = 9 mL/g dry biomass, particle size = -40 mesh, oxygen pressure = 14.8 bar absolute). The pretreated and untreated poplar wood was hydrolyzed simultaneously at 50°C, pH 4.8 for 3 days, using an excess cellobiase loading (i.e., 28.4 CBU/g dry biomass) and various cellulase loadings (i.e., 0, 1, 3, 5, 10, 25, 50, 75 FPU/g dry biomass). The rotating speed of the air shaker was 100 rpm and the concentration of the biomass slurry was 5% w/v. The 3-day glucose and xylose yields were measured using HPLC, whereas the 3-day reducing sugar yields were determined by the DNS assay (see Appendix E for step-by-step procedures). Total sugar yields are calculated as the summation of glucose and xylose yields. The reported sugar yields were calculated by subtracting the corresponding sugar content of the enzymes from the measured sugar yields.

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[5] $$7.5 \text{ g dry biomass} \times \frac{28.4 \text{ CBU}}{1 \text{ g dry biomass}} \times \frac{1 \text{ mL}}{250 \text{ CBU}} = 0.852 \text{ mL}$$

[6] $$7.5 \text{ g dry biomass} \times \frac{75 \text{ FPU}}{1 \text{ g dry biomass}} \times \frac{1 \text{ mL}}{89.7 \text{ FPU}} = 6.271 \text{ mL}$$

Results and Discussions

Fig. 13 shows the 3-day reducing sugar yields from the enzymes alone. The reducing sugar yield from cellulase was 3.3 mg eq. glucose/mL and that from cellobiase was 7.7 mg eq. glucose/mL. At our standard enzyme loadings (0.418 mL cellulase plus 0.852 mL cellobiase, i.e., 5 FPU cellulase plus 28.4 CBU cellobiase per gram dry biomass) for a general 3-d enzymatic hydrolysis, the reducing sugar yield resulting from the enzymes is 7.9 mg eq. glucose/g dry biomass.

The 3-day reducing sugar, glucose, xylose, and total sugar (i.e., glucose plus xylose) yields of untreated and pretreated poplar wood are plotted against cellulase loadings, as shown in Fig. 14. For cellulase loadings larger than 25 FPU/g dry biomass, the sugar yields remain essentially constant. Although the data indicate that, at a cellulase loading of 25 FPU/g dry biomass, the cellulose sites are likely saturated by the enzyme, a cellulase loading of 5 FPU/g dry biomass seems to be sufficient from a practical viewpoint because it represents the "shoulder" of the curve. In Fig. 14, the glucan and xylan conversions presented on the right axis were calculated based on poplar wood compositions reported in "Material Balances."

The sugar yields of untreated poplar wood are significantly lower than those for pretreated samples. Thus, oxidative lime pretreatment causes a dramatic increase in biomass digestibility. At high cellulase loadings (e.g., 75 FPU/g dry biomass), the 3-day glucan conversion of pretreated poplar wood increases about 6 times (i.e., from 16% to 100%), the 3-day xylan conversion increases about 4 times (i.e., from 21% to 88%), and the 3-day total sugar yield increases about 5 times (i.e., from 18% to 97% of theoretical).

From Figure 14, the reducing sugar yields were higher than the total sugar yields. The discrepancy between the reducing sugar measurements and the total sugar measurements probably results because of inaccuracies associated with expressing xylose as equivalent glucose (Chang *et al.*, 1996a).

Conclusions

A cellulase loading of 5 FPU/g dry biomass is selected as the recommended enzyme loading.

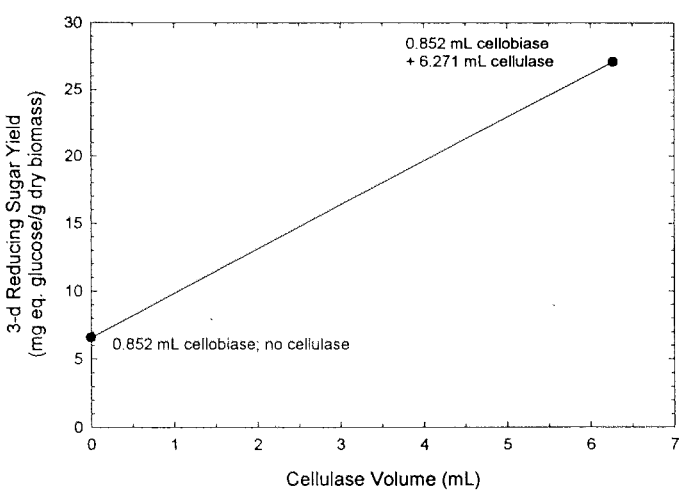
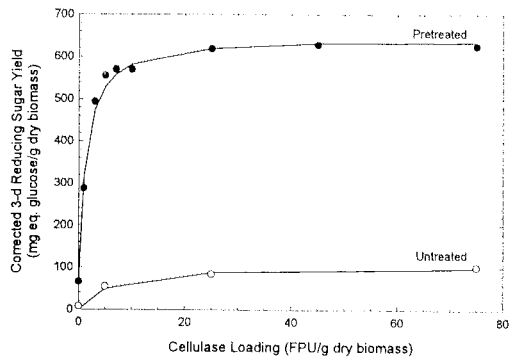
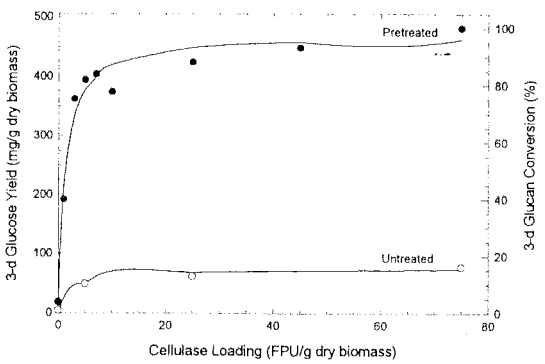


Figure 13. Three-day reducing sugar yields resulting from the enzymes alone



(a)



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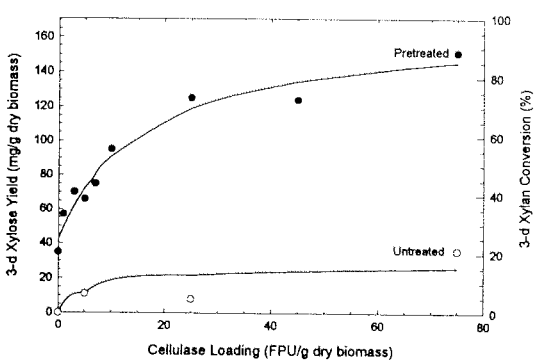

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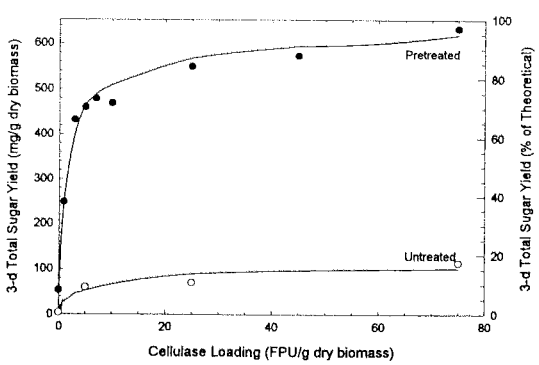


(d)

Figure 14. Enzyme loading studies: (a) 3-d reducing sugar yields; (b) glucose yields; (c) 3-d xylose yields; (d) 3-d total sugar yields
(Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass)

Enzymatic Hydrolysis Profiles

Purpose

Instead of measuring just the 3-day reducing sugar yields, a complete hydrolysis profile is measured so the sugar yields are determined as a function of time.

Materials and Methods

Poplar wood (-40 mesh) was pretreated at 150°C for 6 hours in the presence of 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, and oxygen pressure = 14.8 bar absolute. The pretreated poplar wood was then transferred from the reactors to Erlenmeyer flasks for enzymatic hydrolysis. The hydrolysis conditions were: temperature = 50°C, pH = 4.8, cellulase loading = 5 FPU/g dry biomass, cellobiase loading = 28.4 CBU/g dry biomass, rotating speed = 100 rpm. Samples were withdrawn as a function of time (i.e., 0, 1, 3, 6, 10, 16, 24, 36, 48, 72 h). Reducing sugars were measured using the DNS assay; glucose and xylose were measured using HPLC. This experiment was performed in triplicate. See Appendices B, C, and E for procedures of lime pretreatment, enzymatic hydrolysis, and sugar measurements, respectively.

Results and Discussions

Fig. 15 shows the yields of glucose, xylose, total sugar, and reducing sugars at different hydrolysis times, for untreated and pretreated poplar wood samples. The total sugar yield denotes the summation of glucose and xylose yields. No cellobiose was detected. Unlike the case of switchgrass where the hydrolysis does not proceed significantly further after 24 h, the hydrolysis profiles of pretreated poplar wood show a tendency to proceed further at 72 h. It indicates that hydrolysis times longer than 3 days may result a significant increase in sugar yields.

The sugar yields of pretreated poplar wood are significantly higher than untreated poplar wood. The 3-day reducing sugar yield of pretreated poplar wood increased about 10 times (i.e., from 56 to 565 mg eq. glucose/g dry biomass), the 3-day total sugar yield increased about 12 times (i.e., from 6% to 77% of theoretical), the 3-day glucan conversion increased about 11 times (i.e., from 7% to 77%), and the 3-day xylan conversion increased about 37 times (i.e., from 2% to 74%).

Again, the total sugar yields are smaller than the reducing sugar yields. The discrepancy between the reducing sugar measurements and the total sugar measurements probably results because of inaccuracies associated with expressing xylose as equivalent glucose (Chang *et al.*, 1996a).

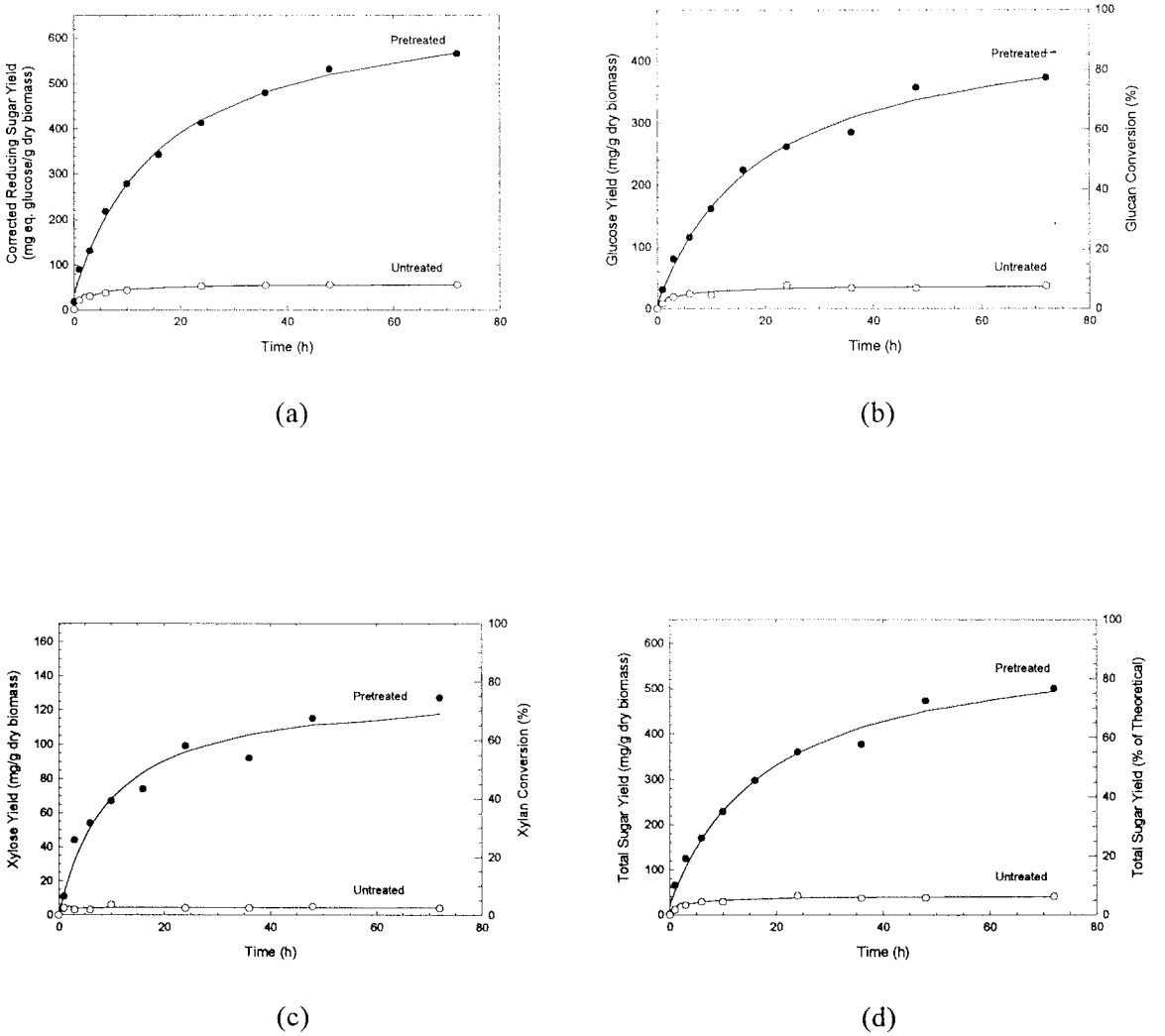


Figure 15. Enzymatic hydrolysis profiles: (a) reducing sugar yields; (b) glucose yields; (c) xylose yields (d) total sugar yields

(Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass)

Enzymatic Saccharification Studies

Purpose

The maximum digestibility of pretreated poplar wood was determined by applying an excess cellulase loading and a long hydrolysis time.

Materials and Methods

Sample Preparation

A large quantity (ca. 45 g dry weight) of poplar wood was pretreated under the recommended conditions (i.e., 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute). The pretreated poplar wood was washed with distilled water until the decant water became colorless. The pretreated-and-washed poplar wood was filtered through Whatman 934/AH glass fiber filter paper and squeezed to remove the major part of water. A small part (ca. 5 g dry weight) of the squeezed poplar wood was dried at 105°C overnight to determine its moisture content. The rest of the sample was then used in the enzymatic saccharification study and simultaneous saccharification/fermentation (SSF).

The glucan content of α-cellulose (Sigma) was determined using the NREL standard procedure No. 002. The glucan content of α-cellulose is 76.14%.

Enzymatic Saccharification

An amount of pretreated-and-washed poplar wood (equal to the equivalent of 0.1 g cellulose) was weighed according to the results of the material balances (see "Material Balances") and moisture determination (see above, "Sample Preparation"). The weighed biomass, 5 mL citrate buffer (0.1 M, pH 4.8), 400 μg tetracycline, and 300 μg cyclohexamide were added to a 20-mL glass scintillation vial. About 4.43 mL distilled water was added to the vial to make the total volume 10 mL. An excess cellulase (Spezyme, filter paper activity = 59 FPU/mL) loading of 60 FPU/g cellulose (equivalent to 26 FPU/g dry raw biomass) was added. The vial was then incubated at 50°C and 68 rpm for 7 days. The glucose concentration of the 7-d sample was measured using the HPLC. The glucose yield of each sample is calculated as follows:

$$\text{Glucose Yield (\%)} = \frac{\text{glucose concentration (mg/mL)} \times 10\ \text{mL} \times 0.9}{0.1\ \text{g cellulose added} \times 1000\ \text{mg/g}} \times 100 \qquad (2)$$

The NREL standard procedure No. 009 was exactly followed in this experiment. The experiment was performed in duplicate.

Results and Discussions

Table 12 shows the 7-day glucose yields of pretreated-and-washed poplar wood and α-cellulose. The α-cellulose was almost 100% digested after a 7-day hydrolysis period, whereas the digestibility of pretreated-and-washed poplar wood was close to 91%. Compared to switchgrass (Chang *et al.*, 1996a), the 7-d glucose yield of poplar wood increased about 22%. The increase of glucose yield may result from the addition of oxygen in the lime pretreatment.

Table 12. Enzymatic Saccharification of Poplar wood

Substrate	7-day Glucose Yield		
	Sample 1	Sample 2	Average
Pretreated-and-washed Poplar Wood	90.04%	91.28%	90.66%
α-cellulose	99.97%	------	99.97%

Simultaneous Saccharification/Fermentation (SSF)

Purpose

This study is to determine the initial hydrolysis rates and the ethanol yields from pretreated-and-washed poplar wood under simultaneous saccharification/fermentation conditions.

Materials and Methods

Sample Preparation

The sample used in this experiment was prepared as described in "Enzymatic Saccharification Studies."

Determination of Initial Hydrolysis Rate

Approximately 8.6 g wet pretreated-and-washed poplar wood (equal to the equivalent of 2 g cellulose) was hydrolyzed at 37°C, pH 5.0 for 24 hours with a cellulase (Spezyme, filter paper activity = 59 FPU/mL) loading of 25 FPU cellulase/g cellulose (equivalent to 10.85 FPU/g dry raw biomass) under aseptic conditions. The total working volume was 200 mL so the initial cellulose concentration was 1%. The concentrations of glucose and cellobiose were measured as a function of time (i.e., 0, 1, 3, 6, 12, 24 h) using an HPLC equipped with a Biorad HPX-87H column. The glucose yield is calculated as follows:

$$\text{Glucose Yield} = \frac{[\text{Glucose}] + 1.053\,[\text{Cellobiose}]}{1.111 f_G\,[\text{Biomass}]} \times 100 \qquad (3)$$

where [Glucose] = glucose concentration (mg/mL)
[Cellobiose] = cellobiose concentration (mg/mL)
[Biomass] = dry biomass concentration at the beginning of the hydrolysis (mg/mL)
f_G = glucan fraction in dry biomass

The NREL standard procedure No. 008 was exactly followed. The same procedure was also applied to the hydrolysis of untreated poplar wood and α-cellulose. This experiment was performed in triplicate.

Simultaneous Saccharification/Fermentation

Approximately 13 g wet pretreated-and-washed poplar wood (equal to the equivalent of 3 g cellulose) was transferred to a 250-mL Erlenmeyer flask equipped with a glass bubble trap. The cellulase (Spezyme, filter paper activity = 59 FPU/mL) loading was 25 FPU/g cellulose (equivalent to 10.85 FPU/g dry raw biomass) and the concentration of the yeast (*Saccharomyces cerevisiae* D_5A) inoculum was 10% v/v. The total working volume was 100 mL so the initial cellulose concentration was 3%. The

concentrations of glucose, cellobiose, and ethanol were measured as a function of time (i.e., 24, 48, 72, 96, 120, 144, and 168 h) using an HPLC equipped with a Biorad HPX-87H column. The ethanol yield is calculated using the following formula:

$$\text{Ethanol Yield} = \frac{[\text{EtOH}]-[\text{EtOH}]_i}{0.568 f_G\,[\text{Biomass}]} \times 100 \quad (4)$$

where

$[\text{EtOH}]_i$ = ethanol concentration at the beginning of SSF (mg/mL)
[EtOH] = ethanol concentration at a time point (mg/mL)
[Biomass] = dry biomass concentration at the beginning of SSF (mg/mL)
f_G = glucan fraction in dry biomass

The NREL standard procedure No. 008 was exactly followed. The same procedure also applied to untreated poplar wood and α-cellulose. This experiment was performed in triplicate.

Results and Discussions

Determination of Initial Hydrolysis Rate

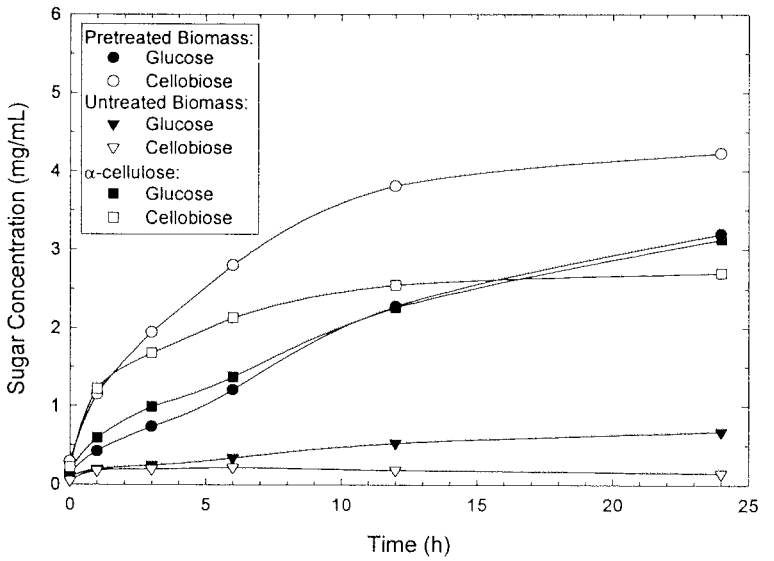
Fig. 16a shows the concentrations of glucose and cellobiose at different hydrolysis times, whereas Fig. 16b shows the glucose yields as a percentage of the theoretical yields during hydrolysis. In the cases of pretreated biomass and α-cellulose, the cellobiose concentrations increased faster than glucose concentrations in the beginning of hydrolysis; after 12 h of hydrolysis, the cellobiose concentrations increased only slightly whereas the glucose concentration still tended to increase, as shown in Fig. 16a. The cellobiose and glucose concentrations of untreated poplar wood were much lower than those of pretreated poplar wood and α-cellulose.

The glucose yields from pretreated poplar wood were slightly higher than those of α-cellulose, as shown in Fig. 16b. It resulted from the higher cellobiose concentrations of pretreated poplar wood whereas the glucose concentrations of pretreated poplar wood and α-cellulose were approximately equal. The 24-h glucose yields were 66% for pretreated poplar wood, 7% for untreated poplar wood, and 51% for α-cellulose. The 24-h pH of each sample was about 4.85 and no contamination was observed on the YPD plates for all samples afterwards.

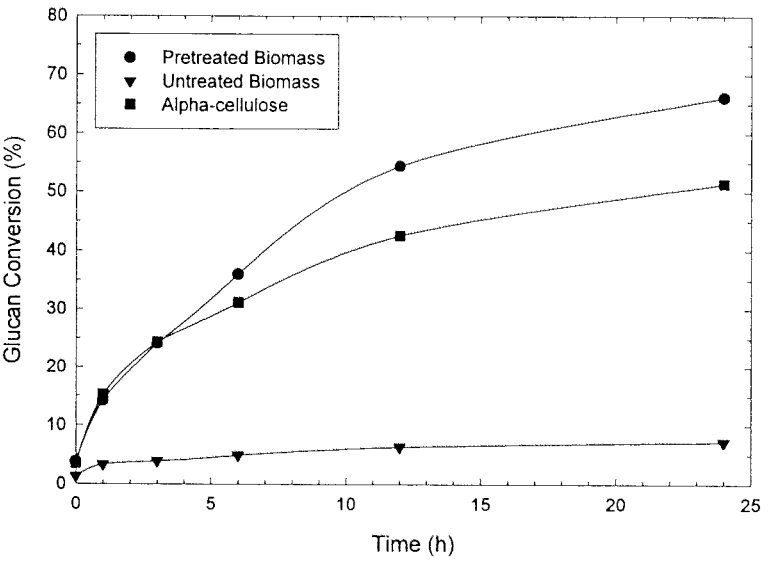
Simultaneous Saccharification/Fermentation

Fig. 17a shows the glucose and cellobiose concentrations of pretreated poplar wood, untreated poplar wood, and α-cellulose during SSF. The cellobiose concentrations were much lower than glucose concentrations in all three cases. In the cases of α-cellulose, no cellobiose was detected after 3 days, whereas for untreated poplar wood, no cellobiose was found throughout the whole SSF period. The glucose concentrations of pretreated poplar wood and α-cellulose remained approximately constant after 2 days whereas those of untreated poplar wood were approximately constant throughout 7 days.

Fig. 17b and 17c show the ethanol concentrations and ethanol yields, respectively. The ethanol yields from pretreated poplar wood increased drastically during the first two days of SSF and increased only slightly after two days. The highest ethanol yield of pretreated poplar wood was 73% and occurred at day 5. Similarly, α-cellulose produced the major part of ethanol during the first two days and its ethanol yield also reached the highest value (i.e., 85%) at day 5. Compared to pretreated poplar wood and α-cellulose, the ethanol yields of untreated poplar wood were fairly low and did not increase significantly after day 1; its highest ethanol yield was only 17% and occurred at day 3.

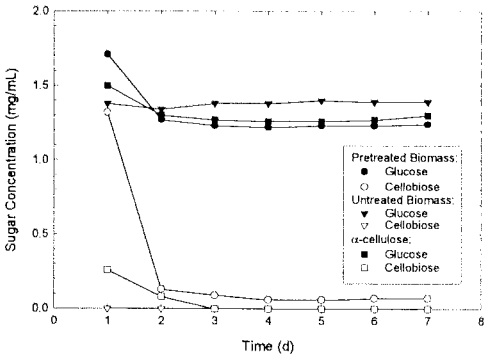


(a)

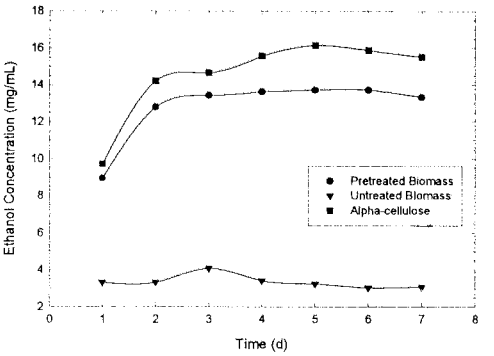


(b)

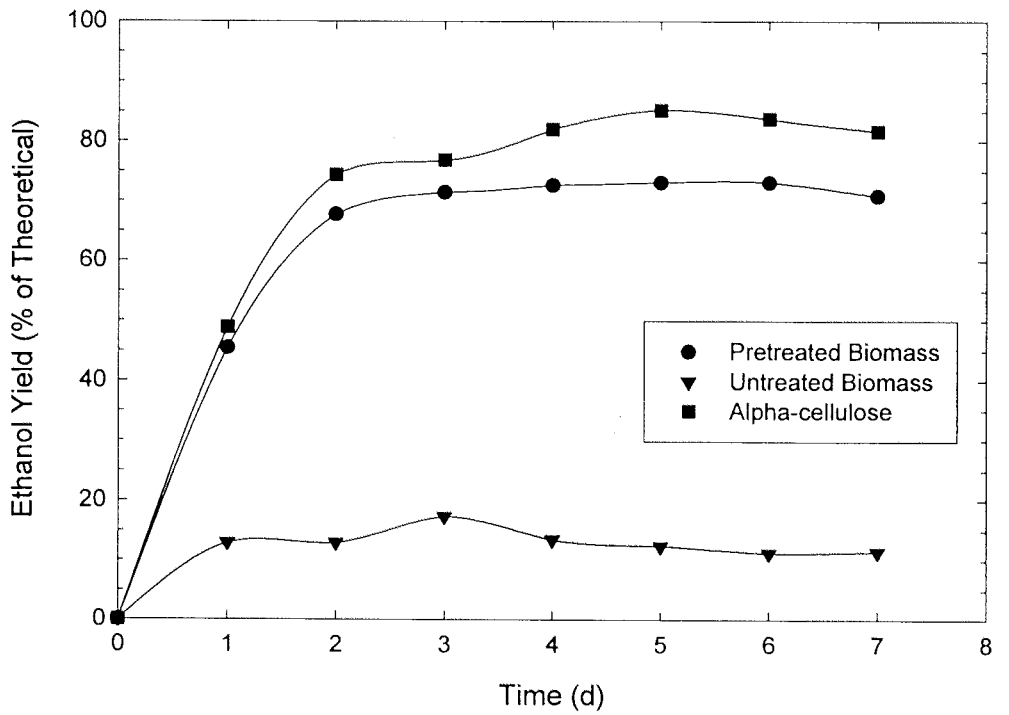
Figure 16. Initial hydrolysis rate study under SSF: (a) sugar concentrations; (b) glucan conversion (Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute)



(a)



(b)



(c)

Figure 17. Simultaneous saccharification/fermentation (SSF):
(a) Sugar concentrations; (b) Ethanol concentrations; (c) Ethanol yields
(Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute)

The yeast culture before inoculation had a dry cell weight of 0.0017 g/mL, an optical density at 600 nm of 0.498 under a dilution factor of 10, a glucose concentration of 24.4 g/L, and an ethanol concentration of 10.91 g/L. The pH readings after 7 days of SSF were 4.35, 4.40 and 4.40 for the three pretreated poplar wood samples, 4.70 for untreated poplar wood, and 4.42 for α-cellulose. After streaking samples on YPD plates and incubating them at 38°C for 24 h, no contamination was observed.

Lime Recovery

Purpose

In the industrial process, lime will be neutralized with carbon dioxide. The pretreated biomass will be washed with water to remove the lime. The wash water will be contacted with carbon dioxide to precipitate calcium carbonate which may be separated from the liquid and converted to lime. This process allows lime to be recycled so that the cost of lime consumption is effectively minimized. This study is to determine how much lime can be recovered after poplar wood is optimally pretreated.

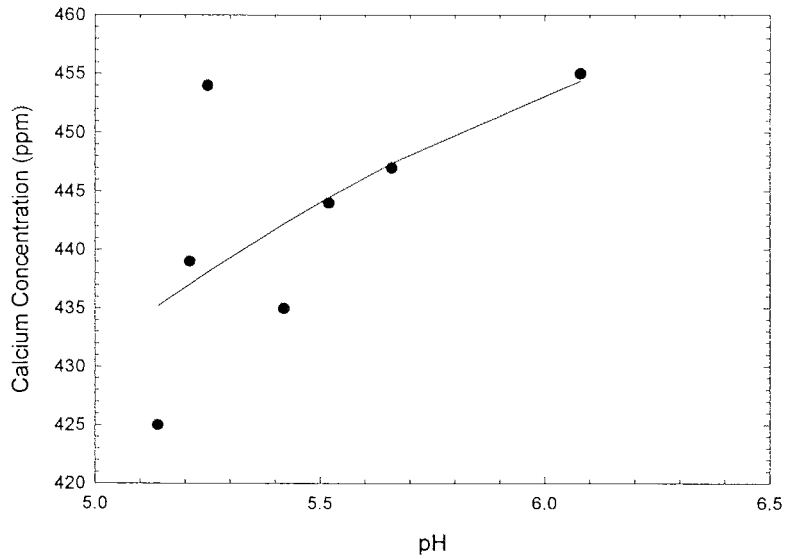
Materials and Methods

A large amount of poplar wood (ca. 30 g) was optimally pretreated (i.e., 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute). The biomass mixture was filtered and the filtrate was transferred to a 500-mL graduated cylinder. The wet biomass was squeezed and the squeezed liquid was collected and mixed with the previous filtrate in the 500-mL cylinder. The total filtrate volume was measured (ca. 270 mL). The squeezed biomass was stored in a 400-mL beaker for further washing. Half of the filtrate (ca. 135 mL) was contacted with carbon dioxide while the pH was monitored. A small amount of sample (ca. 10 mL) was withdrawn and the calcium content was measured as a function of the pH. The carbonation proceeded until the pH of the filtrate could not be further lowered by bubbling carbon dioxide. The pH with the minimal calcium concentration was used as the optimal pH for further precipitation. The other half of the filtrate was contacted with carbon dioxide until the pH dropped to the optimal value. A small liquid sample was withdrawn and the calcium content was measured before and after carbon dioxide contact. The squeezed biomass was mixed with fresh distilled water (ca. 270 mL) and stirred for 10 min. The biomass mixture was filtered and the wet biomass was squeezed. (For each cycle, the squeezed liquid was combined with the filtrate and the mixed liquid was then contacted with carbon dioxide until the pH reached the optimal value as determined previously.) The biomass was repeatedly washed with fresh distilled water for 10 times. Ten milliliters of sample were withdrawn and the calcium content was measured before and after carbonation. The calcium content of the samples were measure using atomic adsorption (see Appendix F).

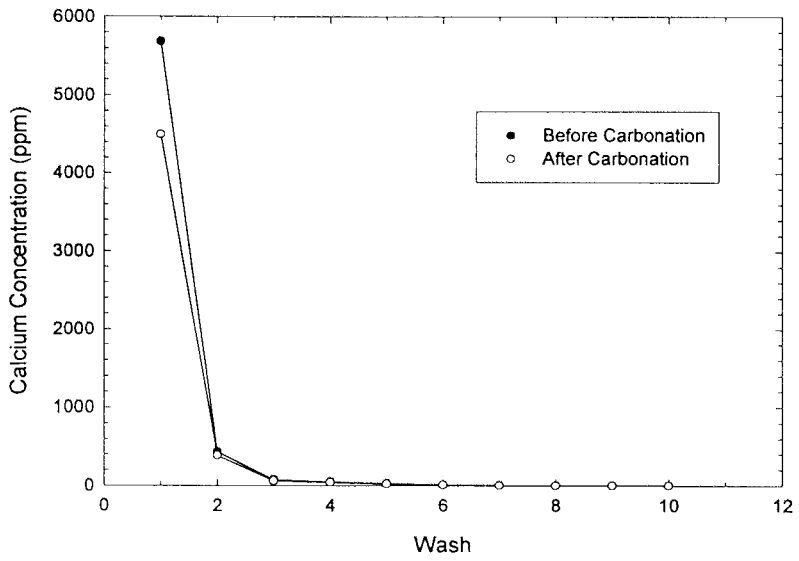
Results and Discussions

The initial pH of the first filtrate was 4.87. After a long period of carbonation (ca. 30 min), the pH remained the same. Therefore, the optimal pH for further precipitation was unable to be determined using the first filtrate. Fig. 18a shows the calcium content of a half of the *second* filtrate as a function of the pH. The lowest calcium concentration occurred at the lowest pH. The curve indicates that the lower the pH, the lower the calcium concentration (i.e., the more $CaCO_3$ precipitated); therefore, the further precipitation proceeded until the pH reached the minimal limit.

Fig 18b shows the calcium content in the wash water before and after carbonation. After 3 washings, no more lime was removed from the pretreated biomass and no more $CaCO_3$ was precipitated (since the two curves coincide). It indicates that it is not necessary to wash the pretreated poplar wood more than three



(a)



(b)

Figure 18. (a) Optimization of the pH; (b) Lime recovery
(Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute)

times to remove and recover the lime. Table 13 shows that, after repeated washings, the filtrate was carbonated allowing about 21% of the added calcium to be recovered as a calcium carbonate precipitate.

Conclusions

Although the calcium recovery of pretreated poplar wood is higher than that of pretreated switchgrass (21% vs. 6%), the conditions required by Process A (Chang *et al.*, 1996a) cannot be met because the amount of $CaCO_3$ precipitate is still too low and too much biomass (ca. 43%) is solublized by the pretreatment. However, the conditions for Processes B, C, and D (Chang *et al.*, 1996a) can be met by poplar wood. In the case of poplar wood, Processes C may be more appropriate than Process B because the pH of the poplar wood slurry after the oxidative lime pretreatment is 4.87, which is very close to the preferred pH for commercial cellulase (i.e., pH 4.8) and the operational pH for SSF (i.e., pH 5.0). Furthermore, about 93% of the recoverable calcium (ca. 20% of the added calcium) was precipitated during the first carbonation (see Table 13); thus repeated washing is not necessary in this case.

Table 13. Lime Recovery for Pretreated Poplar Wood

Calcium input added to 30 g dry raw biomass = 1.6216 g [a]			
Wash	Calcium Content in Filtrate[b]		Ca Recovery[c]
	Before Carbonation	After Carbonation	
1	1.5360 g (94.72%[d])	1.2155 g (74.96%[d])	0.3205 g (19.76%[d])
2	0.1185 g (7.31%[d])	0.1058 g (6.53%[d])	0.0127 g (0.78%[d])
3	0.0219 g (1.35%[d])	0.0178 g (1.10%[d])	0.0041 g (0.25%[d])
4	0.0151 g (0.93%[d])	0.0122 g (0.75%[d])	0.0030 g (0.18%[d])
5	0.0092 g (0.57%[d])	0.0068 g (0.42%[d])	0.0024 g (0.15%[d])
6	0.0049 g (0.30%[d])	0.0041 g (0.25%[d])	0.0008 g (0.05%[d])
7	0.0027 g (0.17%[d])	0.0022 g (0.13%[d])	0.0005 g (0.03%[d])
8	0.0019 g (0.12%[d])	0.0016 g (0.10%[d])	0.0003 g (0.02%[d])
9	0.0016 g (0.10%[d])	0.0014 g (0.08%[d])	0.0003 g (0.02%[d])
10	0.0016 g (0.10%[d])	0.0011 g (0.07%[d])	0.0005 g (0.03%[d])
		Total Recovery =	0.3451 g (21.28%[d])

[a] Equivalent to 3 g $Ca(OH)_2$
[b] Measured using atomic adsorption
[c] Ca recovery = Ca in liquid phase (before carbonation)- Ca in liquid phase (after carbonation)
[d] Based on Ca input (i.e., 1.6216 g)

Conclusions

The results show that lime pretreatment is an excellent pretreatment method. For low-lignin biomass such as switchgrass, the enzymatic digestibility can be significantly increased by lime alone (Chang *et al.*, 1996a, 1996b). For high-lignin biomass such as poplar wood, addition of oxygen is necessary. The 3-d glucan conversion of poplar wood can be increased from 8% to 77% and the 3-d xylan conversion from 2% to 74% by oxidative lime pretreatment, using 5 FPU cellulase/g dry biomass (Fig. 15). At a high cellulase loading (i.e., 75 FPU/g dry biomass), the 3-d total sugar (glucose + xylose) yield was as high as 97% of theoretical. The best ethanol yield we obtained was 73% of theoretical. Lime pretreatment removed about 78% of lignin and 49% of xylan. No glucan was removed by the lime pretreatment. From poplar wood, lime recovery by carbonating wash water is not high. (Our previous study has shown a high lime recovery for other biomass such as bagasse (Nagwani, 1992).) However, three process alternatives are available that overcome this limitation (Processes B, C, and D from Chang *et al.*, 1996a). The recommended conditions for pretreating poplar wood are: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute and a particle size of 20 mesh or smaller.

From a technical viewpoint, lime pretreatment causes high enzymatic digestibility and the simplicity of the process makes it highly applicable. Furthermore, the low cost of lime, the potential ease of lime recovery, and the mild process conditions — which substantially reduce the capital cost — make lime pretreatment a potentially economical process.

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Appendix A. Sample Preparation

Hybrid poplar was used as the substrate in this study, which was provided by the National Renewable Energy Laboratory (NREL). The sample was already air-dried and clean so washing was not necessary. The initial particle size of the material was mainly 20-40 mesh, mixed with sparse wood chops (about 2 mm×10 mm). A portion of the original material was ground using a Thomas-Wiley laboratory mill and sieved through various kinds of sieves according to the required particle sizes. In this study, a particle size of -40 mesh was used in most experiments.

Appendix B. Lime Pretreatment

Lignocellulosic substrate was pretreated with lime (calcium hydroxide) in the presence of water. Six 1.5 in I.D. × 5 in long, 304 stainless steel capped pipe nipples were used as the pretreatment reactors. To ensure thorough mixing of contents inside, the pipe nipples were mounted on a rotating shaft inside an oven providing the desired pretreatment temperature.

To perform the pretreatment, the oven had to be heated in advance to reach the desired temperature. Ground biomass (7.5 g dry weight) and calcium hydroxide (according to the desired lime loading) were placed in each reactor (with the end caps closed) and thoroughly mixed using a spatula. Distilled water (according to the desired water loading) was then added to the dry mixture. After tightly capping the reactors, they were placed in boiling water for 15 min to quickly reach a high temperature and then put into the oven. (For the oxidative lime pretreatments, the reactors were filled with oxygen to the desired pressure at room temperature before being placed in the oven.) The motor was turned on to start the rotating shaft and the reactors were left for the desired pretreatment time. After the pretreatment time elapsed, the reactors were moved out of the oven and immersed in cold water to cool them to ambient temperature. Samples were then removed for enzymatic hydrolysis.

Non-oxidative Lime Pretreatment Procedure

1. Heat the oven to the desired pretreatment temperature. The oven takes about two to three hours to reach a stable temperature. Keep the rotating device inside the oven during heating so that it gets prewarmed.
2. Remove the old Teflon tape and clean the threads at the open end. Wrap clockwise at least four layers of fresh Teflon tape.
3. Label and number all the reactors. Two or four or six reactors can be run each time.
4. Weigh ground and sieved (-40 mesh) material that has 7.5 g dry weight. Using a funnel, pour it in the labeled reactors.
5. Weigh calcium hydroxide, $Ca(OH)_2$, according to the desired lime loading, and pour into the reactors with the biomass.
6. Using a spatula, mix $Ca(OH)_2$ and biomass thoroughly. This dry mixing is essential to ensure a uniform reaction.
7. Pour water according to the desired water loading.
8. Close the reactors by placing the cap on the open end. Hold the nipple in the vice and tighten the cap using a pipe wrench.
9. Place the reactors in boiling water for about fifteen minutes. The water boiler takes about 20 minutes to heat up, so it must be turned on before hand.
10. Place the holders of the reactors in the slots of the rotating rod and tighten the set screws.
11. Place the device in the oven and couple it with the motor using the coupling arrangement.
12. Turn on the motor and keep the rotation speed at the minimum possible. Make sure that the motor does not fall and the motor does not stop rotating. Check periodically during the desired pretreatment time.
13. Observe the oven temperature.
14. After the pretreatment time has elapsed, take out the reactors and place them in a cold tap-water bath. Let them cool for about ten minutes.

Oxidative Lime Pretreatment Procedure

1. Repeat steps 1 to 7 in "Non-oxidative Lime Pretreatment Procedure."
2. Close the reactors by placing the cap (with a "quick connect") on the open end. Hold the nipple in the vice and tightly tighten the cap using a pipe wrench.
3. Make sure the gas regulator on the oxygen cylinder has an enough operating range for the desired oxygen pressure (up to 500 psig) and the tubing and the fittings are appropriate for high-pressure operation (copper tubing is recommended). If not, change it.
4. Connect the "quick connect" with the oxygen cylinder. Open the cylinder valve. If the cylinder gauge shows the oxygen is almost exhausted, a new cylinder must be ordered.
5. Open the regulator valve slowly until the operating gauge reads the desired oxygen pressure.
6. Close the regulator valve and disconnect the "quick connect."
7. Repeat step 9 to 14 in "Non-oxidative Lime Pretreatment Procedure."

Appendix C. Enzymatic Hydrolysis

Lime-pretreated biomass was transferred from the reactors to Erlenmeyer flasks with distilled water. Citrate buffer (1.0 M, pH 4.8,) and sodium azide solution (0.01 g/mL) were added to the slurry to keep constant pH and prevent growth of microorganisms, respectively. Glacial acetic acid was then added to reduce the pH from about 11.5 to 4.8. Then the total volume of the slurry was made up to 150 mL by adding distilled water. The flask was placed in a 100-rpm shaking air bath. When the temperature reached 50°C, cellulase and cellobiase were added to the flask. In the optimization studies, samples were withdrawn after 3 days and then glucose and reducing sugar were measured as 3-day yields. When the hydrolysis profiles were performed, samples were withdrawn as a function of time (i.e., 0, 1, 3, 6, 10, 16, 24, 36, 48, and 72 h) and then sugars were measured at each time point. See the following for the complete hydrolysis procedures. The same procedure was also applied to untreated biomass.

General Enzymatic Hydrolysis Procedure

1. Prepare 250 mL 1-M citrate buffer (pH 4.8) and 100 mL 0.01-g/mL sodium azide solution. (To prepare citrate buffer, first dissolve 210 g of citric acid monohydrate in 1000 mL of distilled water, then adjust the pH to 4.8 by adding NaOH.)
2. Open one end of the reactor and transfer the contents, as much as possible, into a labeled 500-mL Erlenmeyer flask.
3. To completely transfer the biomass, use 50 mL distilled water to wash the reactor. Pour this water and biomass mixture together into the flask.
4. Add 7.5 mL citrate buffer and 5 mL sodium azide solution into the flask to keep the pH constant and prevent the growth of microorganisms, respectively.
5. Add glacial acetic acid to each flask, using a titration tube, until the pH reaches 4.8. During acetic acid addition, continuously monitor the pH and stir using a magnetic bar. Record the volume of acetic acid added. This step should be done very carefully to prevent the pH from dropping below 4.8.
6. Add more water to bring the total liquid volume to 150.0 mL.
7. Place the flask inside the 100-rpm shaking air bath at 50°C.
8. When the temperature reaches 50°C, add 0.418 mL cellulase (filter paper activity ≅ 89.7 FPU/mL enzyme solution) and 0.852 mL cellobioase "Novozyme 188" (activity ≅ 250 CBU/g). This point on the time axis is considered as time zero (time = 0 h)
9. Withdraw 2.0 mL sample using a 5-mL pipet and a cut-off tip. To get a homogeneous sample, shake the flask slightly when withdrawing.
10. Place the flask back into the shaking air bath. Do not tightly cap the flask with a rubber stopper. (Note: This allows the air in the flask to expand as it warms.)
11. Boil samples for 15 min in screw-capped test tubes to denature the enzyme. Cool samples in cold water bath.
12. Close flasks with rubber stoppers after the flasks have been warmed for 10 min.
13. Using a 0.45-μm nylon membrane filter, filter samples into capped sample bottles. Store the samples in the freezer until the DNS assay, YSI glucose analysis, and/or HPLC analysis are ready to be performed. Ensure that each bottle is labeled clearly.
14. Repeat steps 9 to 13 as a function of time (i.e., 1, 3, 6, 10, 16, 24, 36, 48, and 72 h).
15. Perform DNS assay, YSI glucose analysis, and/or HPLC analysis to measure the concentrations of reducing sugars, glucose and/or xylose for each sample.

Appendix D. Biomass Washing Procedure

Washing Procedure for Material Balances between Raw and Washed-Only Biomass

1. Dry about 30 g of untreated biomass at 45°C for 24 hours or longer if necessary.
2. Place and cool the 45°C-dried biomass in the desiccator until it reaches room temperature.
3. Tare a 1-L centrifuge bottle. Transfer and weigh approximately 20 g of 45°C-dried biomass in the centrifuge bottle. Record the weight of the 45°C-dried biomass (W_1).
4. Using the rest of the 45°C-dried biomass, determine the moisture content as described in the NREL standard procedure No. 001 (X_1).
5. Place about 500 mL distilled water in the centrifuge bottle and stir for 15 minutes.
6. Centrifuge the water-biomass mixture at 3300 rpm for 5 minutes. Follow the instruction on the centrifuge machine.
7. During the centrifuge period, setup a vacuum filtration apparatus using a Buchner funnel and a 9-cm Whatman 934/AH glass fiber filter paper (particle retention = 1.5 μm). Weigh the 45°C-dried filter paper before setup. Record the value.
8. After centrifuging, carefully decant the water on the Buchner funnel with vacuum filtration. Decant as much water as possible. Observe the filtrate color.
9. Transfer as much filter cake into the centrifuge bottle as possible.
10. Repeat step 4 through 8 until the filtrate becomes clear. If it takes too long to filter, replace the old filter paper with a new one which has been dried and weighed in advance.
11. After being completely washed, transfer all the biomass in the centrifuge bottle, as well as the filter paper, into a container which has been weighed and recorded. Dry the biomass and filter paper at 45° C for 24 hours or longer if necessary.
12. Place and cool the biomass and filters in the desiccator until it reaches room temperature. Weigh them and record the values (W_2).
13. Using about 5 g of 45°C-dried washed biomass, determine the moisture content as described in the NREL standard procedure No. 001 (X_2). Store the rest of the biomass in the desiccator for determining the contents of ash, lignin, carbohydrate, and protein later.
14. The total weight loss due to washing is calculated using the following formula:

$$\text{Total Weight Loss} = \frac{W_1 \times (1 - X_1) - W_2 \times (1 - X_2)}{W_1 \times (1 - X_1)} \times 100\% \quad (5)$$

where W_1 = 45°C-dried weight of raw biomass (g)
X_1 = moisture content of 45°C-dried raw biomass
W_2 = 45°C-dried weight of washed biomass (g)
X_2 = moisture content of 45°C-dried washed biomass

Washing Procedure for Material Balances between Raw and Pretreated-and-Washed Biomass

1. Dry about 30 g of untreated biomass at 45°C for 24 hours or longer if necessary.
2. Place and cool the 45°C-dried biomass in the desiccator until it reaches room temperature.
3. Weigh approximately 20 g of 45°C-dried biomass in a plastic weighing dish. Record the weight of the 45°C-dried biomass (W_1).

4. Using the rest of the 45°C-dried biomass, determine the moisture content as described in the NREL standard procedure No. 001 (X_1).
5. Under the optimal conditions, pretreat the biomass as explained in Appendix A.
6. Transfer as much pretreated biomass with 500 mL distilled water from the reactors to a centrifuge bottle and stir for 15 minutes.
7. Repeat steps 6 to 13 used in "Washing Procedure for Material Balances between Raw and Washed Only Biomass."
8. The total weight loss due to pretreatment and washing is calculated using the following formula:

$$\text{Total Weight Loss} = \frac{W_1 \times (1 - X_1) - W_2 \times (1 - X_2)}{W_1 \times (1 - X_1)} \times 100\% \qquad (6)$$

where W_1 = 45°C-dried weight of raw biomass (g)
X_1 = moisture content of 45°C-dried raw biomass
W_2 = 45°C-dried weight of pretreated and washed biomass (g)
X_2 = moisture content of 45°C-dried pretreated and washed biomass

Appendix E. Sugar Measurement

Dinitrosalicylic Acid (DNS) Assay

Reducing sugar was measured using the dinitrosalicylic acid (DNS) assay (Miller, 1959). A glucose standard prepared from the YSI 200 mg/dL glucose standard solution was used for the calibration, thus the reducing sugars were measured as "equivalent glucose." The step-by-step procedure is as follows:

DNS Reagent Preparation

1. Dissolve 10.6 g of 3,5-dinitrosalicylic acid crystals and 19.8 g NaOH in 1416 mL of distilled water.
2. Add 306 g Na-K-tartrate (Rochelle salts).
3. Melt phenol crystals under a fume hood at 50°C using a water bath. Add 7.6 mL of phenol to the above mixture.
4. Add 8.3 g sodium meta-bisulfite ($Na_2S_2O_4$).
5. Add NaOH, if required, to adjust the solution pH to 12.6.

DNS Reagent Calibration

1. Using a 200 mg/dL (i.e., 2 mg/mL) YSI glucose standard, prepare 1-mL samples in test tubes according to Table 14.
2. Place 0.5 mL of each sample into test tubes.
3. Dispense 1.5 mL of DNS reagent into each test tube using a 5-mL Brinckmann dispensette. (The following steps should be done under a fume hood to avoid exposure to phenol vapor.)
4. Place the caps on the tubes and vortex.
5. Boil samples in a water bath for 15 minutes.
6. Cool the test tubes for a few minutes. Add 8 mL of distilled water and vortex.
7. Zero the spectrophotometer (Milton Roy, Spectronic 1001) at 550 nm with distilled water (Note: to stabilize the spectrophotometer, it should be turned on for at least 1 h before using).
8. Measure the absorbance.
9. Prepare a calibration curve.

Reducing Sugar Measurement of Samples

1. Filter samples through a 0.45-μm nylon filter.
2. Accordingly, dilute the filtered samples into test tubes such that the sugar concentration lies between 0.2 to 1.0 mg/mL. Vortex the diluted samples.
3. Place 0.5 mL of each diluted sample into test tubes.
4. Repeat steps 3 to 8 used to prepare the calibration curve.
5. Calculate sugar concentration from the absorbance of the samples using the calibration curve.
6. Calculate the reducing sugar yield by following formula:

$$Y = S \times D \times V / W \tag{7}$$

where Y = reducing sugar yield (mg equivalent glucose/ g dry biomass)
S = sugar concentration in diluted sample (mg equivalent glucose/mL)
D = dilution factor
V = working liquid volume (mL)
W = weight of dry biomass (g)

Table 14. Preparation of Glucose Standard Solutions for DNS Assay

Glucose Concentration (mg/mL)	200 mL/dL YSI Standard (mL)	Distilled Water (mL)
0.2	0.1	0.9
0.4	0.2	0.8
0.6	0.3	0.7
0.8	0.4	0.6
1.0	0.5	0.5

HPLC Carbohydrate Analysis

Glucose, xylose, and cellobiose were measured using high performance liquid chromatography (HPLC). A Biorad Aminex HPX-87P column was used in "Material Balances," "Enzyme Loading Studies," and "Hydrolysis Profiles," whereas a Biorad Aminex HPX-87H column was used in "Enzymatic Saccharification Studies" and "Simultaneous Saccharification/Fermentation (SSF)." The instrumental conditions are as follows:

For Biorad Aminex HPX-87P column:

Sample injection volume:	20 μL
Eluant:	0.45-μm filtered reverse osmosis deionized (RODI) water
Flow rate:	0.6 mL/min
Column temperature:	85°C
Detector:	refractive index

For Biorad Aminex HPX-87H column:

Sample injection volume:	20 μL
Eluant:	0.45-μm filtered 0.01N sulfuric acid
Flow rate:	0.6 mL/min
Column temperature:	65°C
Detector:	refractive index

The equipment used in HPLC are as follows:

Pump:	LDC Analytical Pump, constaMetric 3200
Autosampler:	Spectra-Physics, AS100
Column heater:	Jones Chromatography
RI detector:	LDC/Milton Roy, refractoMonitor III
Integrator:	Spectra-Physics, SP4270
RODI water:	NANOpure Ultrapure Water System

Carbohydrate Standard Preparartion

1. Prepare carbohydrate stock solutions: dissolve 0.5 g 45°C-dried carbohydrate (i.e., glucose, xylose, or cellobiose) in a 100-mL volumetric flask with distilled water.
2. Prepare 5 mL standard solutions in test tubes according to Table 15.

3. Place 0.6-1.0 mL standard solutions into autosampler sample vials.
4. Freeze the standard solutions if the analysis will be done later. (Note: Be sure to thoroughly mix the sample after thawing since freezing separates the sugars from the water.)

Table 15. Preparation of Carbohydrate Standard Solutions for HPLC

In "Enzyme Loading Studies" and "Hydrolysis Profiles"		
Carbohydrate Concentration (mg/mL)	5 mg/mL Stock Solution (mL)	Distilled Water (mL)
0.2	0.2	4.8
0.4	0.4	4.6
0.6	0.6	4.4
0.8	0.8	4.2
1.0	1.0	4.0
In "Material Balances," "Enzymatic Saccharification," and "SSF"		
Carbohydrate Concentration (mg/mL)	5 mg/mL Stock Solution (mL)	Distilled Water (mL)
0.5	0.5	4.5
1	1.0	4.0
2	2.0	3.0
3	3.0	2.0
4	4.0	1.0

Equipment Setup

1. Degas the eluant by vacuum-filtering about 4 L RODI water or 0.01 N H_2SO_4 through a 0.45-μm nylon filter. (Note: the eluant must be degassed at least every other day while continuously running for a long period of time.)
2. Connect HPLC equipment according to the Biorad manual, *Guidelines for Use and Care of Aminex Resin Based Columns*.
3. Turn on the pump, the autosampler, the RI detector, the water circulator, and the integrator. Also turn on the built-in refrigerator of the autosampler by loading a user's file (see below, "Autosampler Setup").
4. At a flow rate of 0.2 mL/min, turn on the column heater. Adjust the temperature setting to desired temperature (i.e., 65 or 85°C). Place a mercury thermometer in the column heater as an independent measurement. Usually it needs about 1 h to reach the desired temperature.
5. After the column heater reaches the desired temperature, gradually (i.e., increase 0.1 mL/min every minute) increase the flow rate to 0.6 mL/min. (Note: Do not operate the column at a flow rate greater than 0.2 mL/min at ambient temperature.)
6. Set the cycle time of the integrator to 20 minutes (for "Material Balances," "Enzyme Loading Studies," and "Hydrolysis Profiles") or 30 minutes (for in "Enzymatic Saccharification Studies" and "SSF") by pressing the dialog key and then manually entering the following information:

TIME	FUNCTION	VALUE
TT = 0.0	TF = PM	TV = 1
TT = 20.0 (or 30.0)	TF = ER	TV = 1

For other parameters, just use default values by pressing the enter key.

7. Adjust the energy nob on the detector to 0.5.
8. Press the edit button on the integrator to run a baseline. Adjust the zero nob on the detector to position the base line appropriately. If the baseline is straight and no noise is observed, start running the samples. If not, check if the temperature of the water circulator is constant.
9. Edit and load the autosampler file, as described in "Autosampler Setup."

Carbohydrate Measurement of the Samples

1. Filter the samples through 0.45-μm nylon filters.
2. Dilute the filtered samples such that the carbohydrate concentrations lie between 0.2 to 1.0 mg/mL (for "Enzyme Loading Studies" and "Hydrolysis Profiles"). In "Material Balances," "Enzymatic Saccharification Studies," and "SSF," the original samples were directly detected by HPLC without dilution.
3. Place 0.6-1.0 mL diluted samples into autosampler sample vials.
4. Place the samples and the standard solutions in the autosampler. Edit and load a sample file, as explained in "Autosampler Setup." Adjust the cycle time to 21 min for carbohydrate analysis.
5. Press the run button to start measurements.
6. Collect the chromatograms after all the samples are finished. Prepare a calibration curve according to the chromatograms of standard solutions. Calculate carbohydrate concentrations of the samples according to the calibration curve and the chromatograms of the samples.

Autosampler Setup

Editing/Loading Autosampler Files

1. Press the menu key to display the main menu. Sequentially select FILES, EDIT, and then INJECTION to display the edit menu using the arrow keys to move the cursor and the enter key to select.
2. Adjust the loop size to 20.0 μL and the number of injections per sample to 2 using the "+" or "-" key to increase or decrease the values, respectively. Adjust the cycle time to 21 minutes (for "Material Balances," "Enzyme Loading Studies," and "Hydrolysis Profiles") or 31 minutes (for in "Enzymatic Saccharification Studies" and "SSF").
3. Turn on the built-in refrigerator by pressing the "+" key to switch the option from OFF to ON. Adjust the refrigerator temperature to 5°C using the "+" or "-" key.
4. Use the default values for other parameters in the autosampler file by continuously pressing the enter key.
5. Load the file by selecting FILES and LOAD from the main menu and then pressing the enter key.

Editing/Loading Sample Files

1. Press the sample key to display the sample menu.
2. Specify the sample set number.
3. Adjust the number of injections per sample and the cycle time as explained in "Editing/Loading Autosampler Files."
3. Specify the first sample vial to start with and the number of the samples using the "+" or "-" key.
4. Add the sample set to the queue by pressing the enter key.

Required Maintenance

1. Exactly follow the Biorad instructions to connect the equipment and operate the system. Any deviation from the instruction may destroy the resolution of the column.
2. Distilled water must be the last solvent used before shutting off the pump. Any salts that dry on the plunger will cut through the plunger seals causing wear and eventual leakage.
3. Backwash the column overnight every time when the analysis is finished, according to the Biorad manual, *Guidelines for Use and Care of Aminex Resin Based Columns*. If there are many samples such that it would take more than three days to finish the analysis, backwash the column at least every 48 hours. The operating conditions for backwashing are as follows:

Eluant:	0.45-μm filtered RODI water (Biorad Aminex HPX-87P column)
	0.45-μm filtered 0.01N sulfuric acid (Biorad Aminex HPX-87H column)
Flow rate:	0.1 mL/min
Column temperature:	ambient
Column direction:	reverse

4. Use fresh eluant every time.
5. After the equipment is disconnected, cap the column and guard column with plastic end screws and store them in the refrigerator.

Troubleshooting

1. *Problem*: Unusual long peaks appear in the chromatogram.
   Cause: Air bubbles in eluant are detected by the RI detector.
   Solution:
   a. Replace the eluant with a fresh batch.
   b. Check if there is leaking along the tubes from the pump to the detector. If yes, reconnect the equipment or replace the leaking tube.
   c. Check if the tube connected to the outlet of the RI detector is capillary. If not, replace it with a capillary tube.

2. *Problem*: Carbohydrates do not separate well.
   Cause: The column is losing its resolution.
   Solution:
   a. Check the flow rate and column temperature.
   b. Check if the tubes connecting the autosampler and the guard column, the guard column and the column, and the column and the detector, are all capillary. If not, replace it with a capillary tube.
   c. Check if there is any severe distortion along the tubing. If any severe distortion is observed, replace the distorted tube. (Note: The tubes should be as short and straight as possible, especially for the one connecting the column and the detector.)
   d. Check if a leak occurs along the tubing from the pump to the detector. If yes, reconnect the equipment or replace the leaking tube.
   e. Backwash the column overnight, as explained in "Required Maintenance."
   f. Replace the guard column with a new one.

3. *Problem*: Baseline is out of the scale of the integrator.
   Cause: The temperature in the cell of the RI detector is not stable.
   Solution:
   a. Adjust the zero nob on the detector to position the baseline properly.
   b. Check if the temperature of the water circulator is higher than the ambient temperature. If not, adjust the temperature nob on the water circulator.

c. Check the water level in the chamber of the water circulator. Add water if necessary.

4. *Problem*: Baseline is not stable.
   Cause: Pump pulse is too noisy and/or the reference cell is empty.
   Solution:
   a. Check the pump. Replace the seals and/or add a pulse damper between the pump and autosampler if necessary.
   b. Connect the reference cell to another pump. Flush the reference cell with fresh eluant for at least 5 min (flow rate = 1-2 mL/min). Lower the flow rate to 0.1 mL/min and then push "Standby" button before running samples.

Appendix F. Calcium Analysis

Calcium concentration was measured by the atomic absorption apparatus available in the Kinetics Group of the Chemical Engineering Department of Texas A&M University.

Preparation of Standard Solutions

1. Prepare 20 mg/mL KCl solution: place 10 g KCl in a 500-mL volumetric flask and then add RODI water to a total vomue of 500 mL.
2. Using 1000 ppm calcium reference solution (Fisher Scientific, Lot No. 940982-24), prepare blank and calcium standard solutions in 100-mL volumetric flasks according to Table 14.
3. Transfer the blank and calcium standard solutions to 50-mL disposable polypropylene centrifuge tubes.

Measurement of Samples

1. Filter samples through 0.22-μm nylon filters.
2. Dilute the filtered samples with 10 mL KCl solution and required amount of RODI water in 200-mL volumetric flasks such that the calcium concentrations lie between 1 to 4 ppm.
3. Transfer the diluted samples to 15-mL disposable polypropylene centrifuge tubes.
4. Zero the atomic absorption apparatus with the blank solution.
5. Measure the absorbances of calcium standard solutions. Plot a calibration curve.
6. Measure the absorbances of the samples. Calculate the calcium concentrations of samples using the calibration.

Table 16. Preparation of Blank and Calcium Standard Solutions for Atomic Absorption

Calcium Concentration (ppm)	1000 ppm Calcium Reference Solution (mL)	20 mg/mL KCl Solution (mL)	RODI Water (mL)
Blank	0.0	5.0	95.0
1.0	0.1	5.0	94.9
2.0	0.2	5.0	94.8
3.0	0.3	5.0	94.7
4.0	0.4	5.0	94.6

Appendix G. Ethanol Analysis

The ethanol concentrations of SSF samples were measured using high performance liquid chromatography (HPLC). A Biorad Aminex HPX-87H column was used to separate ethanol and carbohydrates. The instrumental conditions are as follows:

Sample injection volume:	20 μL
Eluant:	0.45-μm filtered 0.01N sulfuric acid
Flow rate:	0.6 mL/min
Column temperature:	65°C
Detector:	refractive index

The equipment used in HPLC are as follows:

Pump:	LDC Analytical Pump, constaMetric 3200
Autosampler:	Spectra-Physics, AS100
Column heater:	Jones Chromatography
RI detector:	LDC/Milton Roy, refractoMonitor III
Integrator:	Spectra-Physics, SP4270
RODI water:	NANOpure Ultrapure Water System

Preparation of Standard Solutions

1. Using pure ethanol (200 proof), prepare standard solutions in 100-mL volumertric flasks according to Table 17.
2. Place 0.6-1.0 mL standard solutions into autosampler sample vials.
3. Freeze the standard solutions if the analysis will be done later. (Note: Be sure to thoroughly mix the sample after thawing since freezing separates the sugars from the water.)

Measurement of Samples

Follow the steps in Appendix E, "HPLC Carbohydrate Analysis."

Table 17. Preparation of Ethanol Standard Solutions

Ethanol Concentration (g/L)	Pure Ethanol (g)[a]
1	0.1
5	0.5
10	1.0
25	2.5

a. Based on a total volume of 100 mL.

Appendix H. Experimental Data

Optimization of Pretreatment Condictions

Table 18. Optimization of Pretreatment Time and Temperature (Non-oxidative Lime Pretreatment)[a,b,c]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Pretreatment Times					
	1 h	3 h	6 h	10 h	16 h	24 h
60	179	204	210	-----	-----	219
80	185	206	232	223	249	258
90	149	180	184	213	197	210
100	169	195	220	240	-----	255
110	197	223	255	255	246	246
120	157	183	213	212	238	238
130	198	226	251	266	272	287
140	197	233	248	257	-----	265
150	224	256	263	275	287	267
160	223	246	257	267	251	255
170	239	261	281	273	249	264
180	228	270	240	231	257	243
190	220	270	231	251	209	183
200	248	266	266	273	-----	12
210	283	233	161	74	-----	8

a. Data for Fig. 2a.

b. Pretreatment conditions: 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, -40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. 3-d corrected reducing sugar yields of untreated biomass = 62 mg eq. glucose/g dry biomass

Table 19. Optimization of Pretreatment Time and Temperature (Non-oxidative Lime Pretreatment)[a,b]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Pretreatment Times		
	15 min	30 min	60 min
210	247	257	299
220	189	241	290
230	221	257	219
240	247	318	244
250	222	301	252

a. Data for Fig. 2b.

b. Pretreatment conditions: 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, -40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

Table 20. Volume of Acetic Acid Required to Neutralize the Biomass Slurry to pH 4.8 before Enzymatic Hydrolysis (Non-oxidative Lime Pretreatment)[a,b,c]

Temp. (°C)	Volume of Acetic Acid (mL/g initial dry biomass) for Various Pretreatment Times					
	1 h	3 h	6 h	10 h	16 h	24 h
60	0.42	0.42	0.42	-----	-----	0.40
80	0.65	0.39	0.36	0.36	0.36	0.32
90	0.65	0.55	-----	0.51	0.48	0.52
100	0.55	0.52	0.47	0.44	-----	0.42
110	0.55	0.51	0.45	0.44	0.42	0.42
120	0.53	0.47	0.45	0.48	0.44	0.41
130	0.52	0.44	-----	0.40	0.37	0.33
140	0.48	0.42	0.40	0.37	0.36	0.35
150	0.47	0.37	0.36	0.32	0.28	0.28
160	0.41	0.35	0.32	0.31	0.25	0.21
170	0.37	0.32	0.29	0.24	0.15	0.08
180	0.37	0.29	0.21	0.04	0.00	0.00

a. Data for Fig. 3.

b. Pretreatment conditions: 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, -40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. Volume of acetic acid required to neutralize the untreated biomass slurry to pH 4.8 = 0.04 mL/g initial dry biomass

Table 21. Optimization of Lime Loading (Non-oxidative Lime Pretreatment)[a,b]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Lime Loadings					
	0 g $Ca(OH)_2$/g dry biomass	0.05 g $Ca(OH)_2$/g dry biomass	0.1 g $Ca(OH)_2$/g dry biomass	0.15 g $Ca(OH)_2$/g dry biomass	0.2 g $Ca(OH)_2$/g dry biomass	0.3 g $Ca(OH)_2$/g dry biomass
200	285	219	236	233	204	180

a. Data for Fig. 4.

b. Pretreatment conditions: 1 h, 9 mL water/g dry biomass, -40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

Table 22. Optimization of Pretreatment Time and Temperature (Oxidative Lime Pretreatment)[a,b,c]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Pretreatment Times					
	1 h	3 h	6 h	10 h	16 h	24 h
120	252	252	291	312	-----	-----
130	257	282	288	343	-----	-----
140	237	272	342	428	513	471
150	298	319	469	355	341	337
160	289	365	331	312	-----	-----
170	306	376	396	363	-----	-----
180	294	322	281	261	-----	-----

a. Data for Fig. 5.

b. Pretreatment conditions: 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 7.9 bar absolute, -40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. 3-d corrected reducing sugar yields of untreated biomass = 62 mg eq. glucose/g dry biomass

Table 23. Optimization of Pretreatment Time and Lime Loading (Oxidative Lime Pretreatment)[a,b,c]

Time (h)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Lime Loadings					
	0 g $Ca(OH)_2$/g dry biomass	0.05 g $Ca(OH)_2$/g dry biomass	0.1 g $Ca(OH)_2$/g dry biomass	0.15 g $Ca(OH)_2$/g dry biomass	0.2 g $Ca(OH)_2$/g dry biomass	0.3 g $Ca(OH)_2$/g dry biomass
3	223	234	319	417	465	507
4.5	340	-----	447	515	508	513
6	358	362	464	502	548	504
7.5	382	422	529	546	560	529

a. Data for Fig. 6.

b. Pretreatment conditions: 150°C, 9 mL water/g dry biomass, -40 mesh, oxygen pressure = 7.9 bar absolute.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. 3-d corrected reducing sugar yields of untreated biomass = 62 mg eq. glucose/g dry biomass

Table 24. Optimization of Oxygen Pressure and Lime Loading (Oxidative Lime Pretreatment)[a,b,c]

Oxygen Pressure (bar absolute)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Lime Loadings					
	0 g $Ca(OH)_2$/g dry biomass	0.05 g $Ca(OH)_2$/g dry biomass	0.1 g $Ca(OH)_2$/g dry biomass	0.15 g $Ca(OH)_2$/g dry biomass	0.2 g $Ca(OH)_2$/g dry biomass	0.3 g $Ca(OH)_2$/g dry biomass
Non-oxidative	256	183	184	212	175	150
7.9	382	422	529	546	560	529
14.8	420	541	622	631	636	609

a. Data for Fig. 7.

b. Pretreatment conditions: 150°C, 7.5 h, 9 mL water/g dry biomass, -40 mesh.
Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. 3-d corrected reducing sugar yields of untreated biomass = 62 mg eq. glucose/g dry biomass

Table 25. Optimization of Oxygen Pressure and Pretreatment Time (Oxidative Lime Pretreatment)[a,b,c]

Time (h)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Oxygen Pressure				
	No extra oxygen	7.9 bar absolute	14.8 bar absolute	21.7 bar absolute	28.6 bar absolute
1	224	243	238	276	281
3	256	368	382	488	507
6	263	467	604	622	605
10	275	551	624	620	608

a. Data for Fig. 8.

b. Pretreatment conditions: 150°C, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, -40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. 3-d corrected reducing sugar yields of untreated biomass = 62 mg eq. glucose/g dry biomass

Table 26. Optimization of Water Loading (Oxidative Lime Pretreatment)[a,b,c]

Temp. (°C)	3-d Corrected Reducing Sugar Yields (mg eq. glucose/g dry biomass) for Various Water Loadings					
	5 mL water/g dry biomass	7 mL water/g dry biomass	9 mL water/g dry biomass	11 mL water/g dry biomass	13 mL water/g dry biomass	15 mL water/g dry biomass
150	449	577	599	595	581	592

a. Data for Fig. 9.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. 3-d corrected reducing sugar yields of untreated biomass = 62 mg eq. glucose/g dry biomass

Table 27. Partical Size Study (Oxidative Lime Pretreatment)[a,b,c]

Partical Size (mesh)	3-d Corrected Reducing Sugar Yield (mg eq. glucose/g dry biomass)
10 to 20	600
20 to 30	597
30 to 40	597
40 to 50	582
50 to 80	561
80 and finer	560
a. Data for Fig. 10.	
b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.	
c. 3-d corrected reducing sugar yields of untreated biomass = 62 mg eq. glucose/g dry biomass	

Material Balances

Table 28. Relative Composition of Raw, Washed-Only, and Pretreated-and-Washed Poplar Wood[a,b]

Components	Contents[c] (%)		
	Raw	Washed-Only	Pretreated-and Washed
Total	100	95.14	57.29
Ash	1.27	0.86	1.10
Lignin	27.91	26.63	4.99
Xylan	15.03	14.23	6.90
Glucan	43.40	42.56	43.36
Crude Protein	1.70	1.32	0.27
Others	10.69	9.53	0.67
a. Data for Fig. 11a.			
b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.			
c. Based on dry weight.			

Table 29. Absolute Composition of Raw, Washed-Only, and Pretreated-and-Washed Poplar Wood[a,b]

Components	Contents[c] (%)		
	Raw	Washed-Only	Pretreated-and Washed
Ash	1.27	0.90	1.92
Lignin	27.91	27.99	8.71
Xylan	15.03	14.96	12.05
Glucan	43.40	44.74	75.68
Crude Protein	1.70	1.39	0.47
Others	10.69	10.02	1.17
a. Data for Fig. 11b.			
b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.			
c. Based on dry weight.			

Table 30. Mineral Contents of Raw, Washed-Only, and Pretreated-and-Washed Poplar Wood[a,b]

Mineral	Contents[c] (%)		
	Raw	Washed-Only	Pretreated-and Washed
S	0.03	0.02	1.90×10^{-3}
B	2.00×10^{-3}	1.60×10^{-3}	7.00×10^{-4}
Ca	0.31	0.32	1.23
P	0.04	0.01	0.00
Fe	0.01	4.50×10^{-3}	0.02
Mg	0.09	0.08	1.80×10^{-3}
Mn	9.00×10^{-4}	9.00×10^{-4}	2.00×10^{-4}
Na	0.02	0.02	0.02
Cu	5.00×10^{-4}	1.10×10^{-3}	0.01
Zn	2.00×10^{-4}	2.00×10^{-3}	2.00×10^{-3}
K	0.29	0.02	0.00
Al	4.00×10^{-4}	3.40×10^{-3}	0.01
Total	0.78	0.49	1.29
a. Data for Fig. 12.			
b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.			
c. Based on dry weight.			

Enzyme Loading Studies

Table 31. Reducing Sugars Resulting from Enzymes[a]

Cellulase Volume (mL)	Cellobiase Volume (mL)	3-d Reducing Sugar Yield (mg eq. glucose/g dry biomass)
0	0.852	6.6
6.271	0.852	27.1

a. Data for Fig. 13.

Table 32. Enzyme Loading Studies[a]

Cellulase Loading (FPU/g dry biomass)	3-d Reducing Sugar Yield (mg eq.glucose/g dry biomass)	
	Untreated Poplar Wood	Pretreated Poplar Wood[b]
0	8	66
1	-----	288
3	-----	494
5	55	555
7	-----	570
10	-----	570
25	84	619
45	-----	628
75	101	625

a. Data for Fig. 14a.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass.

Table 33. Enzyme Loading Studies (for Pretreated Poplar Wood)[a,b]

Cellulase Loading (FPU/g dry biomass)	Glucose[c]		Xylose[c]		3-d Total Sugar Yield[d]	
	3-d Yield (mg/g dry biomass)	3-d Glucan Conversion (%)	3-d Yield (mg/g dry biomass)	3-d Xylan Conversion (%)	(mg/g dry biomass)	(% of theoretical)
0	18	4	35	21	54	8
1	192	40	57	33	249	38
3	362	75	70	41	432	66
5	394	82	66	39	460	70
7	404	84	75	44	479	73
10	374	78	95	55	469	71
25	424	88	125	73	549	84
45	449	93	124	73	573	88
75	482	100	151	88	633	97

a. Data for Fig. 14b, 14c, and 14d.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh. Hydrolysis conditions: 28.4 CBU cellobiase/g dry biomass

c. Measured using HPLC.

d. Total sugar = glucose + xylose

Table 34. Enzyme Loading Studies (for Untreated Poplar Wood)[a]

Cellulase Loading (FPU/g dry biomass)	Glucose[b]		Xylose[b]		Total Sugar Yield[c]	
	3-d Yield (mg/g dry biomass)	3-d Glucan Conversion (%)	3-d Yield (mg/g dry biomass)	3-d Xylan Conversion (%)	(mg/g dry biomass)	(% of theoretical)
0	4	1	0	0	4	1
5	49	10	11	6	60	9
25	62	13	8	5	70	11
75	78	16	36	21	114	18

a. Data for Fig. 14b, 14c, and 14d.

c. Measured using HPLC.

d. Total sugar = glucose + xylose

Enzymatic Hydrolysis Profiles

Table 35. Hydrolysis Profiles [a]

Time (h)	3-d Reducing Sugar Yield (mg eq.glucose/g dry biomass)	
	Untreated Poplar Wood	Pretreated Poplar Wood[b]
0	1	18
1	21	90
3	30	131
6	37	218
10	43	278
16	-----	342
24	52	412
36	54	479
48	56	531
72	56	565

a. Data for Fig. 15a.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

Table 36. Hydrolysis Profiles (for Pretreated Poplar Wood)[a,b]

Time (h)	Glucose[c]		Xylose[c]		3-d Total Sugar Yield[d]	
	3-d Yield (mg/g dry biomass)	3-d Glucan Conversion (%)	3-d Yield (mg/g dry biomass)	3-d Xylan Conversion (%)	(mg/g dry biomass)	(% of theoretical)
0	0	0	0	0	0	0
1	31	6	11	6	65	10
3	81	17	44	26	125	19
6	116	24	54	31	170	26
10	162	34	67	39	229	35
16	224	46	74	43	298	46
24	261	54	99	58	360	55
36	284	59	92	54	377	58
48	357	74	115	67	472	72
72	373	77	127	74	500	77

a. Data for Fig. 15b, 15c, and 15d.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh. Hydrolysis conditions: 5 FPU cellulase/g dry biomass, 28.4 CBU cellobiase/g dry biomass.

c. Measured using HPLC.

d. Total sugar = glucose + xylose

Table 37. Hydrolysis Profiles (for Untreated Poplar Wood)[a]

Time (h)	Glucose[b]		Xylose[b]		3-d Total Sugar Yield[c]	
	3-d Yield (mg/g dry biomass)	3-d Glucan Conversion (%)	3-d Yield (mg/g dry biomass)	3-d Xylan Conversion (%)	(mg/g dry biomass)	(% of theoretical)
0	0	0	0	0	0	0
1	8	2	4	2	11	2
3	19	4	3	2	21	3
6	24	5	3	2	28	4
10	22	5	6	4	28	4
24	37	8	4	2	42	6
36	33	7	4	2	37	6
48	33	7	5	3	38	6
72	37	8	4	2	41	6

a. Data for Fig. 15b, 15c, and 15d.

c. Measured using HPLC.

d. Total sugar = glucose + xylose

Simultaneous Saccharafication/Fermentation

Table 38. Initial Hydrolysis Rate Study of Poplar Wood under SSF[a]

Time (h)	Sugar Concentration (mg/mL)						Glucan Conversion (%)		
	Pretreated[b]		Untreated		α-cellulose		Pretreated[b] Biomass	Untreated Biomass	α-cellulose
	Glu.[c]	Cello.[d]	Glu.[c]	Cello.[d]	Glu.[c]	Cello.[d]			
0	0.14	0.29	0.10	0.05	0.18	0.22	3.80	1.31	3.48
1	0.43	1.15	0.20	0.18	0.59	1.22	14.19	3.32	15.18
3	0.74	1.95	0.25	0.20	0.99	1.68	24.16	3.91	24.35
6	1.21	2.80	0.34	0.22	1.37	2.13	36.01	4.93	31.14
12	2.28	3.81	0.53	0.19	2.26	2.55	54.47	6.38	42.60
24	3.20	4.23	0.68	0.15	3.13	2.70	66.21	7.24	51.41

a. Data for Fig. 16.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh. Hydrolysis conditions: 25 FPU cellulase/g cellulose, no cellobiase.

c. Glucose

d. Cellobiose

Table 39. Sugar Concentrations during SSF of Poplar Wood[a]

Time	Glucose Concentration (mg/mL)			Cellobiose Concentration (mg/mL)		
(d)	Pretreated[b]	Untreated	α-cellulose	Pretreated[b]	Untreated	α-cellulose
1	1.71	1.38	1.50	1.32	0	0.26
2	1.27	1.34	1.30	0.13	0	0.08
3	1.23	1.38	1.27	0.09	0	0
4	1.22	1.38	1.26	0.06	0	0
5	1.23	1.40	1.26	0.06	0	0
6	1.23	1.39	1.27	0.07	0	0
7	1.24	1.39	1.30	0.07	0	0

a. Data for Fig. 17a.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh.

Table 40. Ethanol Concentrations and Yields during SSF of Poplar Wood[a]

Time	Ethanol Concentration (mg/mL)			Ethanol Yield (% of theoretical)		
(d)	Pretreated[b]	Untreated	α-cellulose	Pretreated[b]	Untreated	α-cellulose
1	8.95	3.32	9.72	45.47	12.92	48.86
2	12.80	3.32	14.23	67.73	12.92	74.38
3	13.44	4.08	14.66	71.48	17.34	76.83
4	13.64	3.41	15.58	72.64	13.49	82.02
5	13.73	3.23	16.14	73.11	12.42	85.18
6	13.73	3.02	15.88	73.11	11.23	83.74
7	13.34	3.07	15.52	70.88	11.51	81.69

a. Data for Fig. 17b and 17c.

b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh.

Lime Recovery

Table 41. Optimization of pH for Lime Recovery[a,b]

pH	Calcium Concentration (ppm)
6.08	455
5.66	447
5.52	444
5.42	435
5.25	454
5.21	439
5.14	425
a. Data for Fig. 18a.	
b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh.	

Table 42. Lime Recovery[a,b]

Wash Times	Calcium Concentration (ppm)	
	Before Carbonation	After Carbonation
1	5689	4502
2	439	392
3	81	66
4	56	45
5	34	25
6	18	15
7	10	8
8	7	6
9	6	5
10	6	4
a. Data for Fig. 18b.		
b. Pretreatment conditions: 150°C, 6 h, 0.1 g $Ca(OH)_2$/g dry biomass, 9 mL water/g dry biomass, oxygen pressure = 14.8 bar absolute, -40 mesh.		

ABSTRACT

Lime (calcium hydroxide) was used as the pretreatment agent to enhance the enzymatic digestibility of switchgrass. The effect of lime pretreatment on digestibility at various pretreatment conditions has been studied. The optimal pretreatment conditions were: time = 2 h, temperature = 100°C and 120°C, lime loading = 0.1 g/g dry biomass, water loading = 9 mL/g dry biomass. Studies on the effect of particle size indicate that there was little benefit of grinding below 20-40 mesh; even coarse particles (4-10 mesh) digested well. The effect of cellulase loading for enzymatic hydrolysis has been studied and effective hydrolysis was obtained with 5 FPU/g dry biomass. High sugar yields (79% for glucose and 97% for xylose) were obtained due to the lime pretreatment. Under the optimal pretreatment and hydrolysis conditions, the glucose yield was 3 times that of untreated switchgrass, the xylose yield was 11 times, and the total sugar yield was 4.3 times. Using simultaneous saccharification/fermentation, the best ethanol yield was 70% of the theoretical yield. A material balance study showed that little glucan (ca. 10%) was solublized due to the lime pretreatment whereas about 26% of xylan and 29% of lignin became solublized. The lime could not be recovered by carbonating the wash water because the calcium was complexed with soluble organics. To overcome this limitation, three alternative lime recovery processes are presented.

DEVELOPMENT OF ALTERNATIVE PRETREATMENT AND BIOMASS FRACTIONATION PROCESSES: LIME PRETREATMENT

NREL SUBCONTRACTOR REPORT

Part IV
Laboratory Results for Corn Stover

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Subcontract No.: XAW-3-11181-03
Under Prime Contract DE-AC02-83CH10093

November 1996

ABSTRACT

Native corn stover is resistant to digestion by enzymes. Lime pretreatment is effective for improving the enzymic hydrolysis of corn stover. For industrial purposes, the recommended pretreatment conditions are: lime loading 0.075 g $Ca(OH)_2$/g dry biomass; water loading 5 g H_2O/g dry biomass; and heating for 4 h at 120 °C. Washing the pretreated biomass before neutralization removed about 88% of the lime and reduced the acid required for neutralization by about 50%.

The recommended enzyme loading for the enzymic saccharification of pretreated corn stover is 10 FPU/g dry biomass and the recommended hydrolysis temperature is 40 °C. The enzymic conversion of the corn stover to monosaccharides, when pretreated and saccharified as prescribed, was about 60% of the cellulose, 47% of the xylan, and 53% of the total available polysaccharide. Ultimate digestion of pretreated corn stover produced conversions of 88.0, 87.7, and 92.1% for the glucan, xylan and arabinan, respectively, compared to a glucan conversion of 98.9% for an α-cellulose control. Simultaneous saccharification/fermentation of α-cellulose and pretreated corn stover gave ethanol yields of about 70% of theoretical.

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In the pretreatment of corn stover, five individual parameters were considered: pretreatment time, pretreatment temperature, calcium hydroxide loading, water loading, and biomass particle size. Each parameter was varied in turn while the other four were held constant. The samples generated from permutations in the various inputs were compared using the standard enzymic hydrolysis and dinitrosalycilic acid (DNS) analytical procedures described in Appendix A: Standard Enzymic Procedure and Appendix B: DNS Assay Procedure, respectively.

Determining effective enzymic saccharification conditions is as critical as the pretreatment. The purpose of the present study was to identify the best combination of time, temperature, and enzyme loading for the saccharification of corn stover pretreated with lime at elevated temperature (0.1 to 0.125 g $Ca(OH)_2$/g dry biomass, 10 g H_2O/g dry biomass, 120 °C, 5 h). The effects of using different neutralizing acids and the relative benefit of washing the biomass after pretreatment, but before neutralization, also were examined.

1. Time and Temperature Relationships

1.1. Introduction

Previous studies with bagasse (Nagwani, 1992) showed that time and temperature significantly impact the pretreatment severity and thus represent important processing parameters. Conversely, the lime loading had very little influence on pretreatment severity beyond the optimal value of 0.1 g $Ca(OH)_2$/g dry biomass and the water loading had almost no effect at all. Therefore, for the time/temperature evaluation with corn stover, the recommended pretreatment parameters for bagasse were selected as starting points. These were: calcium hydroxide loading of 0.1 $Ca(OH)_2$ g/g dry biomass; water loading of 10 g/g dry biomass; and particle size of -40 mesh (Nagwani, 1991).

1.2. Materials and Methods

The pretreatments were conducted in reactors constructed from capped, 304 SS, 1.5-in NPT schedule 40, pipe nipples that had a total volume of approximately 200 mL and a working volume of approximately 180 mL. Each sample utilized 7.5 g dry biomass. The calcium hydroxide was added to the dry biomass in each reactor and the contents were mixed. The water was then added to each reactor in two steps. For the first step, all but about 10 mL was added and the slurry was well-mixed. Then, the last 10 mL was used to rinse the solids adhering to the stir-rod into the reactor. The reactor was then tightly closed and heated for the appropriate duration.

The reactors were mounted on a mechanized carriage inside a digitally controlled convection oven; the carriage rotated the reactors during the course of the pretreatment to ensure thorough mixing. The reactors were briefly preheated in a boiling water bath before being placed in the oven to reduce the time necessary to achieve a steady-state treatment temperature.

Temperatures of 100, 120, 130, and 140 °C were explored. Six reactors were utilized for each temperature run; two runs were utilized for 100 °C. The reactors were removed from the oven according to the respective processing times listed in Table 1. Two samples were also prepared that were not pretreated, although lime (0.1 g $Ca(OH)_2$/g dry biomass) was added and immediately neutralized to pH 4.8 with glacial acetic acid.

Table 1. Processing Times for Various Temperatures

100 °C:	3, 6, 7.5, 9, 10.5, 12, 13.5, 15, 16.5, 18, 19.5, 22 (h)
120 °C:	1, 2, 3, 4, 5, 6
130 °C:	1, 2, 3, 4, 5, 6.
140 °C:	1, 2, 3, 4, 5, 6

When each reactor was removed from the oven, it was placed in an ice bath to quench the reaction. The solids were then quantitatively transferred into 500-mL culture flasks. The transfer was conducted with the flask on a digital pan balance so that the total volume of water used to effect the transfer could be carefully monitored; the total mass of pretreated solids and solution was 140 g.

Once the solids transfer was complete, the solutions were prepared for enzymic hydrolysis; the standard enzymic procedure is detailed in Appendix A. In summary: To each flask were pipetted 7.5 mL of 1.0-M citrate buffer (pH 4.8) and 5.0 mL of 10-g/L aqueous sodium azide solution. Then the pH was adjusted to 4.8 using glacial acetic acid and 28.4 IU/g[1] β-glucosidase (Novozyme 188) and 5 FPU/g cellulase (Spezyme-CP) were added. The flasks were then cultured in an incubated shaker (50 °C, 100 rpm, Amerex Instruments Orbital Shaker Incubator #GM706) for 72 h. At the conclusion of the enzymic hydrolysis, 20-mL samples were collected from each flask; the samples were placed in sealed vials and heated in a boiling water bath for 10 min to denature the enzymes. The solutions were then centrifuged (IEC DPR 6000) and refrigerated until ready for analysis by the DNS assay.

The DNS assay is detailed in Appendix B. To summarize: Hydrolyzate was diluted 1:50 with distilled water. Then, 0.5 mL of the diluted solution was pipetted into a 22-mm screw-cap culture tube to which 1.5 mL of DNS reagent was added. This mixture was then placed in a boiling water bath for 15 min, cooled, and diluted with 8 mL of distilled water. The absorbance at 550 nm of the reacted mixture was compared to a calibration curve prepared using glucose standard solutions.

[1] All enzyme loadings given as activity units/gram of dry biomass.

1.3. Results and Discussion

Apparent yields for each of the conditions were calculated from the DNS assay. The results (mg equiv glucose/gram dry biomass) are presented in graphical form in Figure 1. (Note: The 12-h sample for 100 °C was compromised and therefore omitted.) The maximum yield was about 440 to 460 mg equiv glucose/gram of dry biomass, independent of reaction temperature. The average sugar yield for the non-pretreated samples was 54 mg glucose equiv/gram dry biomass.

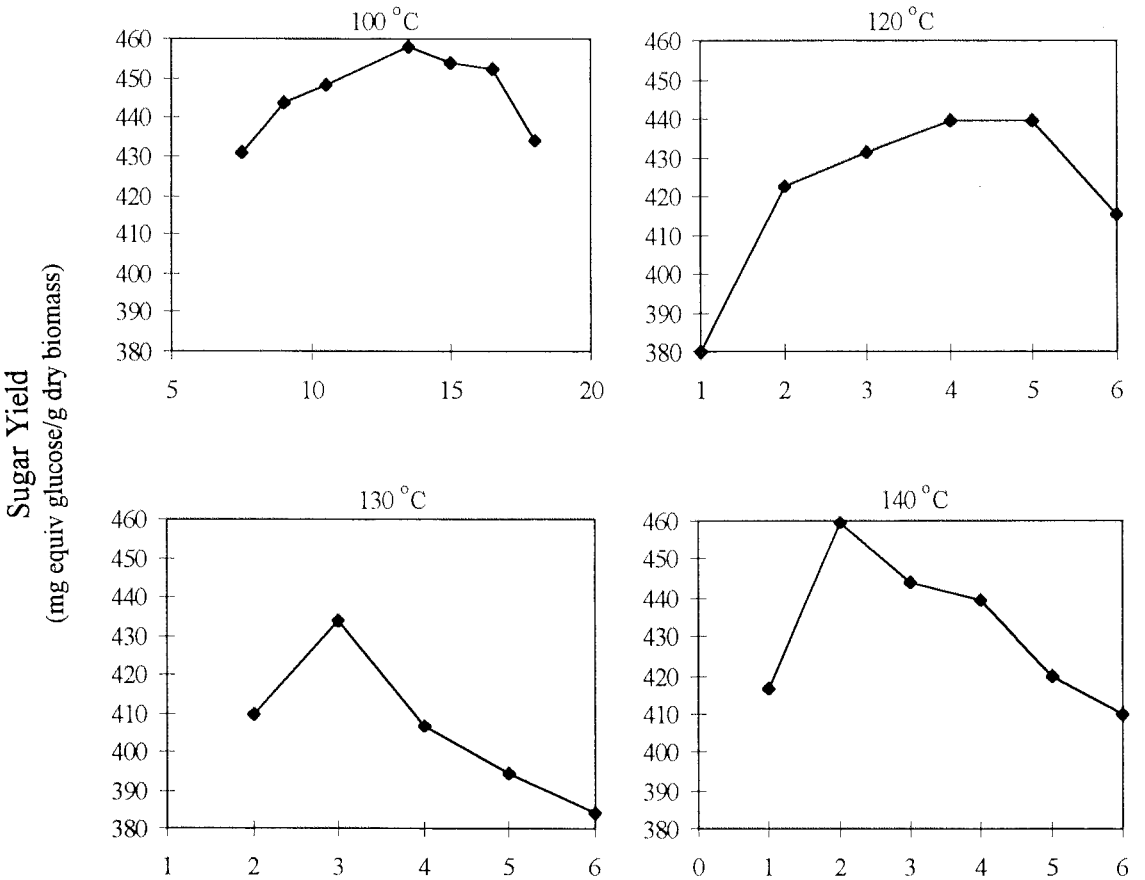

Figure 1. Sugar yields for various pretreatment times and temperatures.

From Figure 1, the optimal treatment time for each temperature is readily identified; these times are: 100 °C, 13.5 h; 120 °C, 4.5 h; 130 °C, 3 h; 140 °C, 2 h. It was found that a semi-log plot of run time vs. treatment temperature was linear (Figure 2; R^2 of 0.993). The optimal treatment time can be correlated using:

$$t = 0.22 \exp -\left(\frac{(T-100)}{20.9}\right) \qquad (1)$$

where t is the treatment time in minutes and T is the reaction temperature in degrees Celsius. This equation is comparable to the "severity index" relationship derived by Overend and Chornet (1987) for the steam explosion process:

$$R_o = t \exp\left(\frac{(T-100)}{14.7}\right) \qquad (2)$$

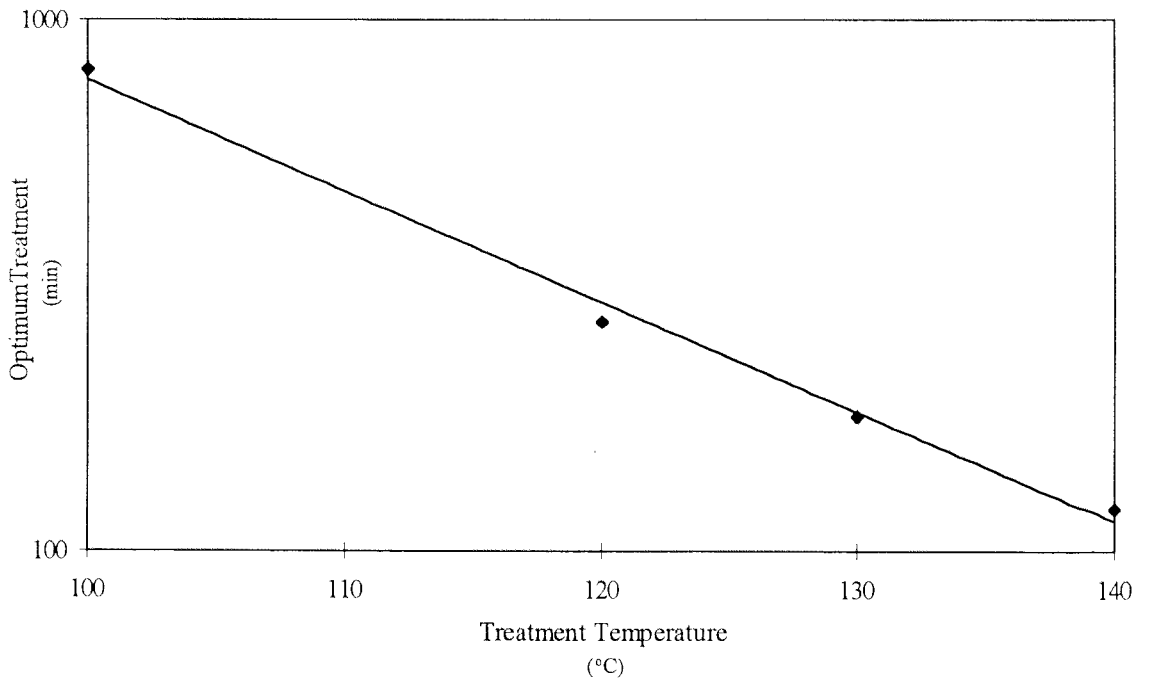

Figure 2. Optimum prediction line for pretreating corn stover.

The selection of an "optimum" treatment temperature for an industrial process requires examining the trade-off between expediency and equipment cost; the higher temperatures (greater than 100 °C) would require costly pressurized equipment. For this reason 120 °C was identified as the "optimum" treatment temperature because the generated steam pressure, 101 kPa, would not be excessive and the treatment time (5 h) would not be much longer than that required for the higher temperatures.

2. Calcium Hydroxide Loading

2.1. Introduction

The most expensive input for the pretreatment of corn stover is the calcium hydroxide. For this reason, experiments were conducted to determine the minimum loading of calcium hydroxide required to effect the pretreatment.

2.2. Materials and Methods

An identical procedure to that used for the time and temperature studies was utilized except the calcium hydroxide loading was varied while the treatment time and temperature were held constant at 5 h and 120 °C, respectively. The calcium hydroxide loadings used were 0.0, 0.01, 0.025, 0.075, 0.10, 0.125, 0.15, 0.175, 0.20, 0.25, and 0.30 g $Ca(OH)_2$/g dry biomass; the volumes of glacial acetic acid required to neutralize the pretreated biomass to pH 4.8 are plotted in Figure 3.

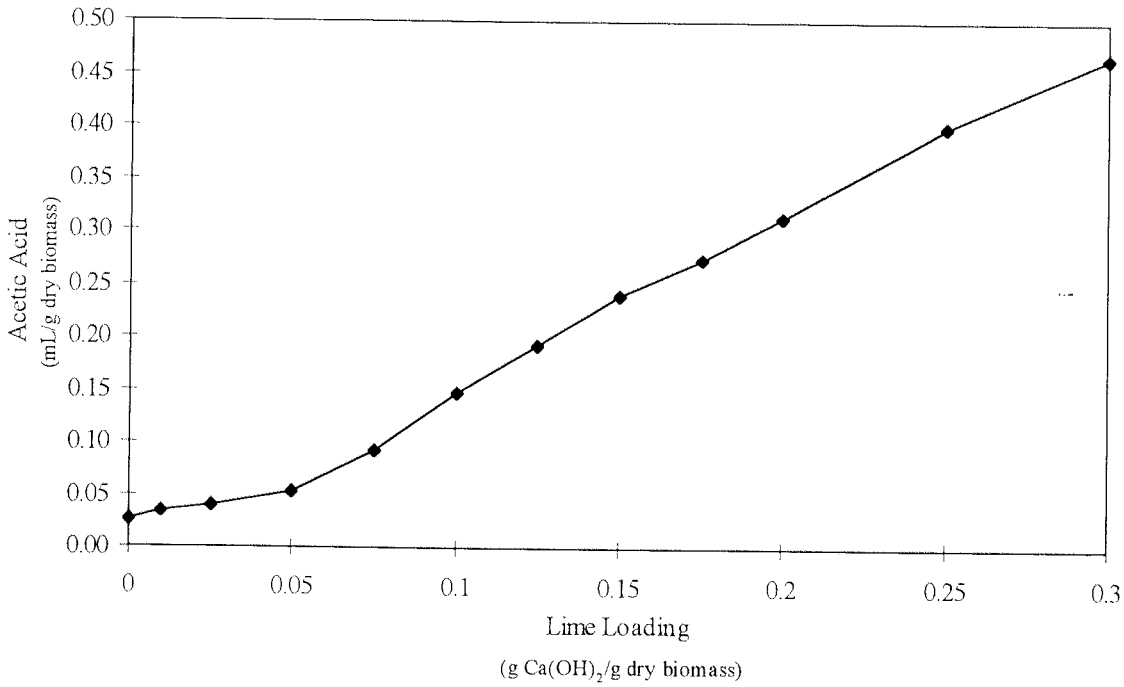

Figure 3. Glacial acetic acid required to neutralize pretreated corn stover to pH 4.8.

2.3. Results and Discussion

The sugar yields increased from 182 mg equiv glucose/g dry biomass (no lime) to about 462 mg equiv glucose/g dry biomass (lime loadings between 0.1 and 0.25 g $Ca(OH)_2$/g dry biomass); no advantage was observed for increasing the lime loading above 0.1 g $Ca(OH)_2$/g dry biomass (Figure 4). A recommended lime loading would be approximately 0.075 g $Ca(OH)_2$/g dry biomass because the yield obtained at this loading was nearly as high as that achieved with 0.1 g $Ca(OH)_2$/g dry biomass, yet 25% less lime was used.

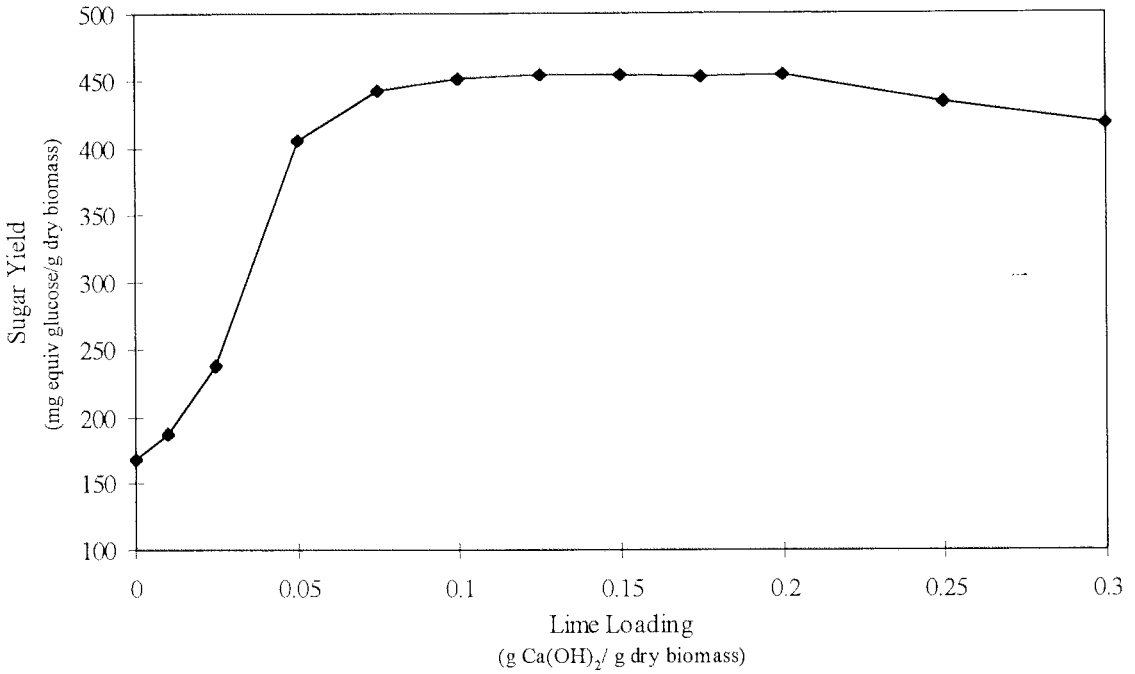

Figure 4. Sugar yield for various calcium hydroxide loadings.

3. Water Loading

3.1. Introduction

The water added to the corn stover should be the least necessary to effect the pretreatment. Though water itself is not expensive, the associated equipment and energy costs associated with its introduction into the system make it an important variable in the economics of the industrial process. Therefore, a wide range of water loadings were investigated; the resulting mixtures varied from thick pastes to thin slurries.

3.2. Materials and Methods

Corn stover samples (-40 mesh, 7.5 g dry fiber) were pretreated with aqueous calcium hydroxide solution at 120 °C for 5 h using a procedure identical to that used for the time/temperature studies. The water loading was varied from 5 to 15 g H_2O/g dry biomass, in 1 g H_2O/g dry biomass increments. The same calcium hydroxide loading (0.125 g $Ca(OH)_2$/g dry

biomass) was used for all samples. This lime loading was selected because it is on the plateau of the pretreatment curve (Figure 4) and removed from the shoulder occurring around 0.075 $Ca(OH)_2$/g dry biomass.

3.3. Results and Discussion

The pretreatment was independent of water loading. The average sugar yield for the 12 samples (two at 10 g H_2O/g dry biomass) was 442 $\pm$ 5.7 mg equiv glucose/g dry biomass with a range of 430-449 mg equiv glucose/g dry biomass. It is probable that water loadings of less than 5 g H_2O/g dry biomass would still allow for effective pretreatment provided the reaction vessel could be adequately mixed.

4. Particle Size

4.1. Introduction

Wilke et al. (1981) estimated that about 7% of the cost of obtaining glucose from corn stover by enzymic hydrolysis following a dilute acid pretreatment could be attributed to milling the corn stover to pass a 2-mm screen (10 mesh). Because milling cost is directly related to the target particle size, it was important to ascertain whether any pretreatment advantage existed for small particle sizes.

4.2. Materials and Methods

Using standard stainless steel wire sieves, the milled corn stover was manually size-classified into the following size ranges: +20 mesh; -20 to +40 mesh; -40 to +80 mesh; and -80 mesh. The majority of the material fell into the -20+40 classification. Identical pretreatment conditions (7.5 g biomass, 0.125 g $Ca(OH)_2$/g dry biomass, 10 g H_2O/g dry biomass, 120 °C, 5 h) were used to treat six samples from each size category; the lime loading was selected by the same

rationale as in Section 1.3. The pretreated samples were hydrolyzed and analyzed by DNS assay according to standard conditions.

4.3. Results and Discussion

The sugar yields for the samples are given in Table 2. As can be seen, mesh size had no effect on the conversion. The low value for the first sample listed in the -20+40 range was due to a titration error which resulted in a low pH during the enzymatic hydrolysis; the result from this sample was not used in the calculations. The lower values for the -80 mesh material are likely due to a higher content of impurities (dirt, etc.) in this fraction. Based on these results, it is possible that an industrial process could successfully use large (greater than 20 mesh) particle sizes, or perhaps even unmilled corn stover.

Table 2. Sugar Yield for Various Mesh Sizes of Corn Stover

+20 mesh	-20+40 mesh	-40+80 mesh	-80 mesh
443 mg/g	385 mg/g (dis.)	435 mg/g	394 mg/g
448	438	407	392
440	440	435	394
438	438	432	400
418	438	452	402
411	413	430	389
433 $\pm$ 14.9	433 $\pm$ 11.4	432 $\pm$ 14.5	395 $\pm$ 4.9

5. Lime Recovery

5.1. Introduction

Recovering the lime used for the pretreatment is a critical factor for the economics and environmental impact of the future industrial process. Ideally, the lime is extracted from the pretreated biomass with water and carbon dioxide is used to precipitate calcium carbonate from the wash. An experiment was conducted to examine the feasibility of this process approach.

5.2. Materials and Methods

Milled, unsieved, corn stover (750 g dry) was placed in an 8-L stainless steel beaker. Calcium hydroxide (75 g) was added to the stover and the contents of the beaker was well-mixed. Distilled water (3.75 L) was then added to the dry material and the resulting paste was stirred until homogeneous. A stainless steel lid was placed on the beaker which was then put into a large autoclave. The temperature in the autoclave was brought to 122 °C using live steam. After 2.5 h at 122 °C, the autoclave was vented to atmospheric pressure and the beaker containing the pretreated corn stover was removed. The corn stover was then allowed to cool at room temperature; the material at the center of the beaker remained hot even after 3 h on the bench. The pretreated corn stover was transferred from the beaker to an airtight plastic jar and stored at 4 °C until ready for use. The solids content of the bulk pretreated corn stover, determined by drying at 105 °C, was 19.5%.

Aliquots of the pretreated corn stover (42.3 g), equivalent to 7.5 g dry fiber and 0.75 g calcium hydroxide, were weighed into three tared 1-L centrifuge bottles. Distilled water (150 mL) was added to each of the samples, the lids were attached, and the bottles were placed in an orbital shaker (150 rpm) for 15 min. The bottles were then removed from the shaker, a minimal amount of water was used to rinse the interior walls, and the samples were centrifuged (3500 rpm) for 5 min.

The supernatants were carefully decanted into separate tared 1-L centrifuge bottles. After removing 1-mL samples from each supernatant, carbon dioxide was introduced into the solutions using 7/8- x 1-in. cylindrical porous-silica bubblers until the pH of the solutions were reduced to at least 5.7-5.9; no precipitate was observed. A 1-mL sample was obtained and the neutralized solutions were transferred to a third set of tared 1-L centrifuge bottles.

The washing procedure was repeated with fresh distilled water five times; a 1-mL sample was removed after each wash. After neutralization, the second and third washes were added to the previous wash for the respective samples. Washes 4 and 5 were reused to extract more calcium hydroxide from the biomass samples; Wash 4 was recycled for Washes 6 and 8, whereas Wash 5 was recycled for Wash 7. In all cases, 1-mL samples were obtained from the recycled washes after each stage. The pH of Wash 8 was observed to only increase from 5.9 to 6.5 after contacting the biomass. At this point, it was concluded that additional washing of the biomass would be ineffective; the washing procedure was discontinued and Washes 7 and 8 were added to the composite wash for each respective sample.

The volumes of the composite washes were determined before the solutions were dried at 105 °C; the residual fiber solids were also dried at 105 °C. The calcium content of the 1-mL samples was determined by atomic adsorption spectroscopy (Varian Spectra AA-30) after diluting the samples 1/400.

5.3. Results and Discussion

The pertinent data from the experiment is listed in Table 3. Notably, the calcium concentrations of the first washes before (-P) and after (-C) carbonation are essentially the same; this result substantiates the observation that little or no calcium carbonate precipitated. The average total calcium recovery from the extraction procedure was 82.4% of the original loading. The residual solid contained 2.1% calcium, compared to 0.3% for the raw biomass. Thus, 12.8% of the added calcium remained in the pretreated biomass, leaving approximately 5% not accounted for; the missing calcium was probably lost in small precipitates that formed in the sample vials before the washes were analyzed.

Table 3. Data from Extraction of Pretreated Corn Stover

Wash Stage	Sample 1			Sample 2			Sample 3			Average [Ca]	Avg. Cumul.
	[Ca] (g/L)	pH-P	pH-C	[Ca] (g/L)	pH-P	pH-C	[Ca] (g/L)	pH-P	pH-C	(g/L)	Ca Recovery[d]
1-P[a]	0.701	11+	5.75	0.699	11+	5.75	0.707	11+	5.75	0.702	0.313
1-C[b]	0.664	----	5.75	0.734	----	5.75	0.648	----	5.75	0.682	----
2-P	0.232	11+	5.65	0.225	11+	5.65	0.219	11+	5.65	0.225	0.419
3-P	0.131	11+	5.60	0.147	11+	5.60	0.148	11+	5.60	0.142	0.485
4-P	0.081	nd[c]	5.30	0.107	nd	5.40	0.199	nd	5.35	0.106	0.532
5-P	0.064	nd	5.20	0.091	nd	5.20	0.068	nd	5.20	0.074	0.567
6-P	0.260	7.0	5.90	0.265	7.0	5.85	0.256	7.0	5.85	0.260	0.648
7-P	0.249	6.5	5.70	0.263	6.5	5.70	0.242	6.5	5.70	0.251	0.737
8-P	0.319	6.6	5.90	0.344	6.6	5.85	0.328	6.6	5.85	0.330	0.824
comp-C	0.341	----	5.70	0.346	----	5.70	0.328	----	5.70	0.338	----

Key: [a]Precarbonation; [b]carbonated; [c]not determined; [d]g Ca/g Ca loaded.

The average cumulative calcium recovery is plotted as a function of washing stage in Figure 5; this figure illustrates some unexpected results.

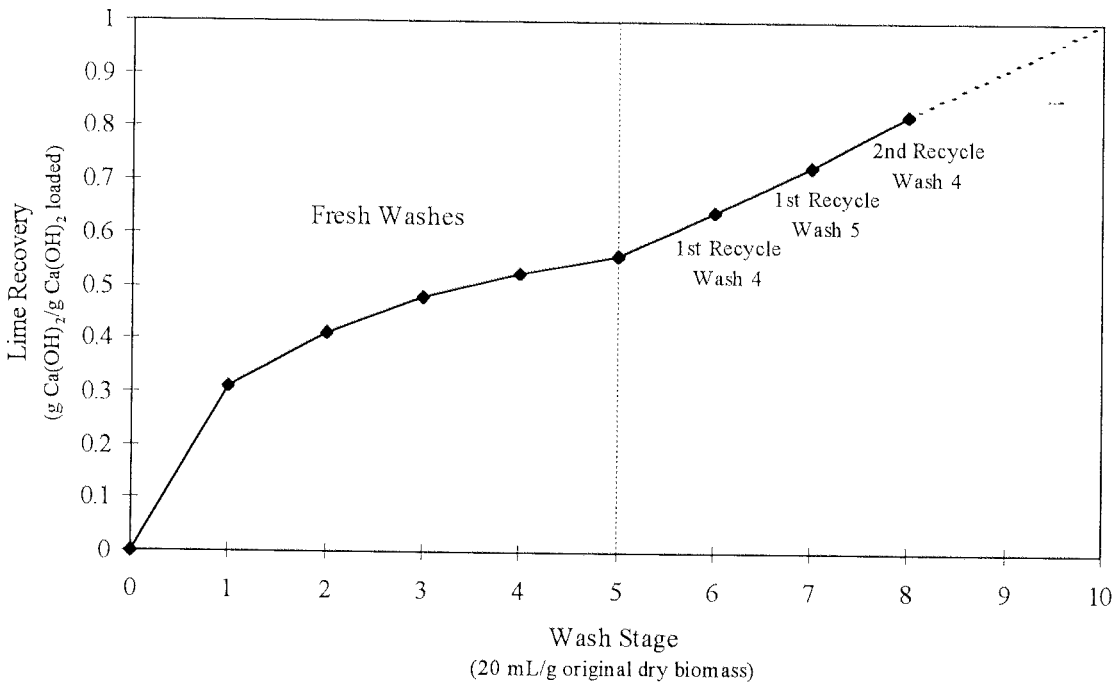

Figure 5. Progress of lime recovery by batch extraction of pretreated corn stover.

It is apparent that the pH change of the wash water after an extraction stage was not a good measure of washing effectiveness. The (positive) linear calcium recovery curve from Wash 5 to Wash 8 shows that the washing procedure was working well and should have been continued beyond Wash 8. Furthermore, the relative performances of Washes 4, 6, and 8 indicate that carbonated wash water is more effective at extracting lime from pretreated corn stover than fresh distilled water despite the preexisting calcium carbonate in the wash. It is likely that nearly all of the lime could have been extracted from the biomass in as few as two additional stages if the washing procedure had continued (Figure 5). Removing all of the lime from the pretreated biomass is advantageous because the acid required for the pH adjustment for the subsequent

hydrolysis and/or fermentation of the biomass is greatly reduced (see Section 6). However, washing the biomass does result in lower sugar recovery (see Section 6).

The results from the extraction experiment suggest a change to the recommended procedure for recovering the lime. A possible configuration for an industrial process would have two counter-current extraction stages using fresh water in the first stage and carbonated water in the second; the wash would not be carbonated after extracting the biomass. The wash water could be concentrated using process heat obtained by combusting the non-fermentable solids; the concentrate would then be fed to the kiln to retrieve the lime.

6. Effects of Washing Pretreated Biomass Before Neutralization and Hydrolysis

6.1. Introduction

One means to reduce the acid required to neutralize the pretreated biomass is to wash the biomass after pretreatment, thereby removing some of the alkali. The consequence of the washing is that some of the carbohydrates in the biomass will also be removed, thus reducing the yield of fermentable sugars. An experiment was conducted to quantify the effects of post-pretreatment washing.

6.2. Materials and Methods

Four corn stover samples were pretreated according to the standard conditions (0.1 g $Ca(OH)_2$/g dry stover, 10 g H_2O/g dry stover, 120 °C, 5 h). One of the samples was transferred to a 500-mL culture flask by methods described previously. The other three samples were quantitatively transferred to 1-L centrifuge bottles. The solids in the centrifuge bottles were separated from the liquid fractions by centrifugation for 5 min at 3500 rpm. The supernatants were decanted, 150 mL of distilled water was added to each flask, and the solids were agitated

with a stirring rod. The centrifugation step was repeated, and the supernatants were discarded as before. This washing step was conducted a total of ten times for each sample. The ten-stage washing procedure was also performed on two 7.5-g corn stover samples that had not been pretreated.

After the last aliquot of wash was discarded, the solids from two pretreated samples and the two non-pretreated samples were placed in a drying oven at 105 °C to determine the weight loss. After determining the net dry mass, portions of these dry samples were acid hydrolyzed according to the method described in Appendix D and analyzed by HPLC by the methods detailed in Appendix C.

The other washed pretreated sample was quantitatively transferred to a 500-mL culture flask with water such that the total mass in each flask was 138 g. After adding citrate buffer and sodium azide solution as usual, the washed sample in the culture flask, and the sample that had not been washed, were neutralized to pH 4.8 with glacial acetic acid. The washed sample required 0.5 mL of acetic acid to obtain pH 4.8, compared with 1.1 mL for the unwashed sample. The two samples were then hydrolyzed by routine for 100 h at 40 °C. At the conclusion of the hydrolysis 20-mL aliquots were removed, placed in a boiling water bath for 10 min, and analyzed using the DNS assay.

6.3. Results and Discussion

The average weight loss by washing for the two non-pretreated samples was 12.0%; the weight loss was 23.5% for the two pretreated samples. The compositions of these washed samples, as well as that for the raw milled corn stover, are listed in Table 4.

Table 4. Compositions of Various Corn Stover Samples

Sample	Glucan	Xylan	Arabinan	Total Sugars	Lignin[a]	Ash	Protein
Untreated	39.0	20.1	2.0	61.1	21.5	6.8	3.9
Washed	42.7	20.6	2.0	65.3	21.7	3.8	2.5
	(37.8)	(18.2)	(1.8)	(57.5)	(19.1)	(3.3)	(2.2)
P/T & Washed	47.6	18.7	1.8	68.1	18.9	8.4	1.9
	(40.1)	(15.7)	(1.5)	(57.3)	(15.9)		(1.6)

Note: ( ) indicates value as percent of original, untreated, biomass. [a]Includes acid soluble and insoluble lignins, corrected for ash.

Table 4 shows that the washing and pretreatment operations concentrated some polysaccharides, particularly glucan, but always reduced the total amount of polysaccharides compared to the untreated biomass weight. The reduction is readily apparent by examining the enzymic hydrolysis data in which the difference between the unwashed and washed conversions was 6.13%. Therefore, the ultimate decision as to wash the pretreated biomass before neutralization would depend on the relative cost of the acid compared to the combined cost of the sugars and the washing stage. This relationship would vary depending on the feedstock and the plant location.

7. Neutralizing Agents

7.1. Introduction

Ideally, an industrial process would utilize carbon dioxide gas to directly neutralize the lime after the pretreatment forming calcium carbonate precipitate in the hydrolytic solution. However, many fermentation organisms that are currently being utilized for biomass conversion have pH optimums that are lower than can be obtained with carbon dioxide; the minimum pH attainable with carbon dioxide is approximately 5.5. The optimum pH for brewer's yeast, for example, is around 3.75 (Taylor, et al., 1995) whereas the optimum for genetically engineered

Klebsiella oxytoca is about 5.0 (Burchhardt and Ingram, 1992). The neutralization of lime with carbon dioxide has other potential problems: (1) low solubility of CO_2 in water; (2) mass transfer resistance of CO_2 from the gas phase to the liquid phase; (3) low solubility of lime in water; and (4) mass transfer resistance of solid lime dissolution. Therefore, the efficacy of using other acids for the neutralization was investigated. The preferred properties of the neutralizing agent are low cost, formation of an insoluble calcium salt, little or no inhibition of the enzymes, and potential for lime recovery. The three acids selected for study were acetic, sulfuric and phosphoric.

7.2. Materials and Methods

Three corn stover samples were pretreated according to the standard procedure (0.1 g $Ca(OH)_2$/g dry stover, 10 g H_2O/g dry stover, 120 °C, 5 h) and transferred to 500-mL culture flasks. The samples were neutralized to pH 4.8 by titration with either glacial acetic acid (1.1 mL), concentrated phosphoric acid (0.8 mL), or concentrated sulfuric acid (0.3 mL), and enzymes were added according to the procedure described in Appendix B. The samples were then hydrolyzed for 100 h in the shaker-incubator at a temperature of 40 °C. At the conclusion of the hydrolysis, 20-mL aliquots were removed from each sample, the aliquots were placed in a boiling water bath for 10 min, and the samples were analyzed by the DNS assay.

7.3. Results and Discussion

No appreciable difference in sugar yield among the acid types was observed in the samples; the range of yields was 541-552 mg equiv glucose/g dry biomass. Therefore, the best acid for the neutralization could be selected based on the other criteria. With regard to price, sulfuric acid is substantially cheaper than phosphoric acid and acetic acid; Table 5 contains the pertinent cost data (prices from Chemical Marketing Reporter). Both sulfuric acid and

phosphoric acid form insoluble calcium salts, but only acetic acid provides for a method of lime recovery (eq 3).

$$Ca(CH_3COO)_2 \xrightarrow{\Delta} CaCO_3 + CH_3COCH_3 \quad (3)$$

Strictly on the basis of cost, sulfuric acid was identified as the acid of choice. Although the relative values of calcium sulfate and calcium phosphate, or the disposal of these salts and the associated environmental concerns, might dictate the selection of another candidate, it is extremely unlikely that the cost advantage of sulfuric acid could be eroded.

Table 5. Relative Costs of Using Different Neutralizing Acids

Type	Cost ($/kg)	Volume (mL) [a]	Density [b]	Mass (g) [a]	$/ 100 kg biomass
CH_3COOH	0.73	1.1	1.049	1.154	11.23
H_3PO_4	0.75	0.8	1.685	1.348	13.48
H_2SO_4	0.08	0.3	1.84	0.552	0.59

[a] for 7.5 g pretreated biomass ;[b] from Merck Index (1989)

8. Enzymic Saccharification

8.1. Introduction

The most critical factor of the enzymic saccharification is the enzyme loading. Experiments were conducted to identify the optimum enzyme loading for saccharifying pretreated corn stover.

8.2. Materials and Methods

The corn stover samples were pretreated according to the established standard condition: 7.5 g dry biomass pretreated with 0.1 g $Ca(OH)_2$/g dry biomass and 10 g H_2O/g dry biomass for 5 h at 120 °C. The pretreated samples were transferred to 500-mL culture flasks by the procedure described previously and citrate buffer (1.0 M, 7.5 mL) and sodium azide (10 g/L, 5 mL) solutions were added. The pH was adjusted to 4.8 using glacial acetic acid before β-glucosidase

(28.4 IU/g), and cellulase were added; the cellulase loadings were 1, 3, 5, 7, 10, and 15 FPU/g dry biomass. The hydrolysis was conducted at 40 and 50 °C. For both experiments, 4-mL aliquots were removed at time intervals of 1, 3, 6, 10, 16, 24, 36, 48, 72, and 100 h; the samples were sealed in vials, heated in a boiling water bath for 10 min, centrifuged, and the supernatants analyzed by the DNS assay.

8.3. Results and Discussion

The results of the experiments are plotted in Figure 6; this figure contains some important features. First and foremost, higher conversions were obtained at 40 °C. A key to the increased conversions can be can be seen in the slopes of the conversion lines for the lower enzyme loadings. The slopes of the conversion lines for the 1 and 3 FPU/g dry biomass samples at 40 °C are positive after 100 h indicating that the enzymes were still active. These slopes can be compared to the flat conversion lines for all of the 50 °C samples. However, plotting the data as a function of enzyme loading (Figure 7) reveals that, despite the increased performance at 40 °C, the optimal enzyme loading, identified as the shoulder of the yield curve, for hydrolysis at 40 °C is comparable to that for hydrolysis at 50 °C; the loading should be about 10 FPU/g dry biomass.

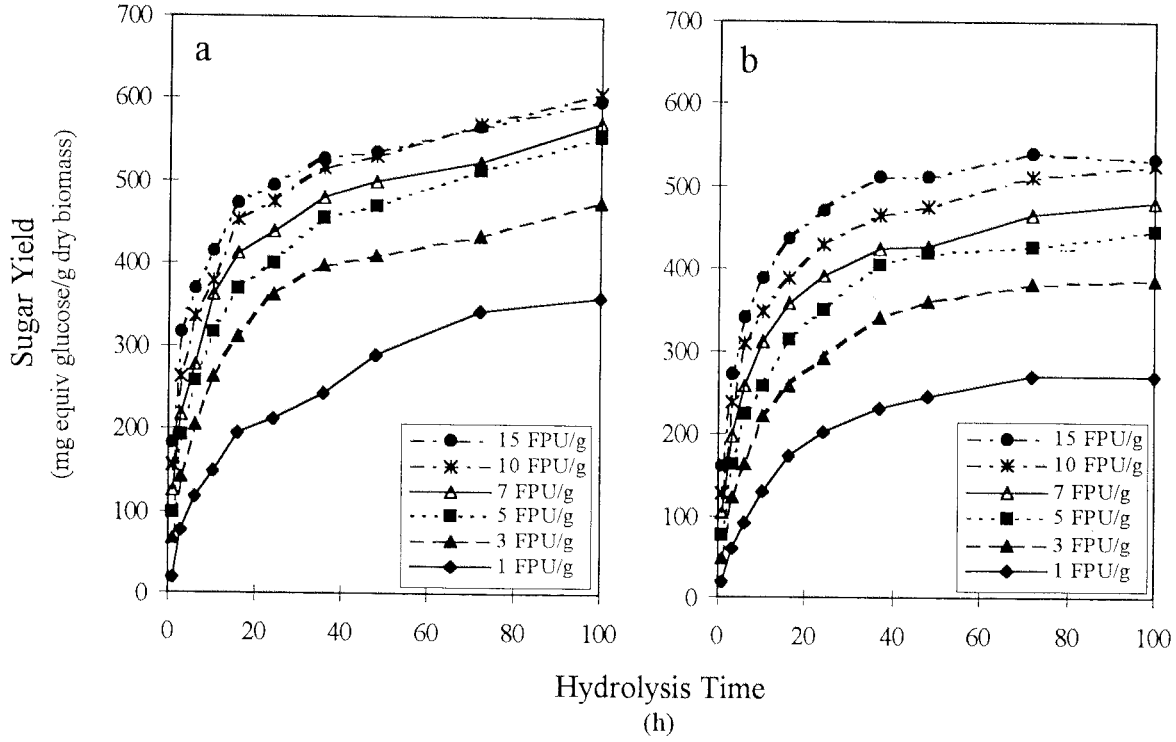

Figure 6. Sugar yield as a function of enzyme loading: (a) 40 °C and (b) 50 °C.

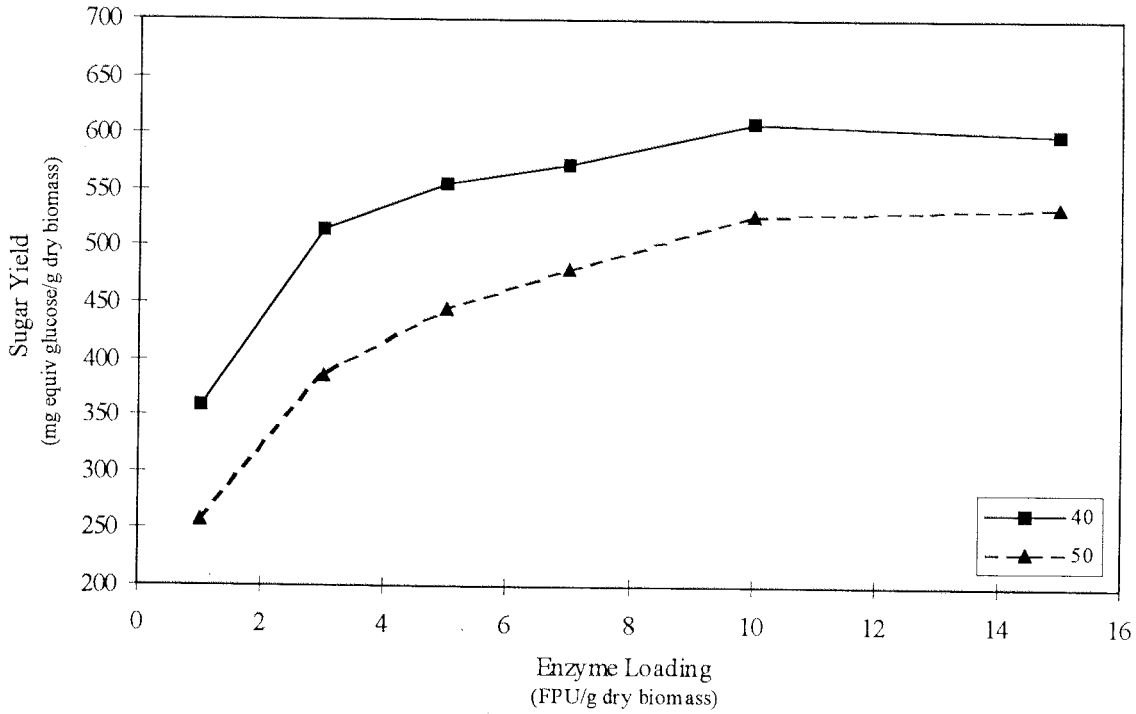

Figure 7. Sugar yields for different enzyme loadings after 100 h of hydrolysis at 40 and 50 °C.

9. Ultimate Digestion

9.1. Introduction

The ultimate digestion is performed with high enzyme loadings and long hydrolysis times and is typically used to compare different biomass pretreatment processes (Torget, 1993).

9.2. Materials and Methods

Two corn stover samples were prepared by the established procedure (7.5 g dry biomass, 0.1 g $Ca(OH)_2$/g dry biomass, 10 g H_2O/g dry biomass, 120 °C, 5 h) and quantitatively transferred to 500-mL culture flasks by methods described previously. Two α-cellulose samples were also prepared in 500-mL culture flasks using 4.0 g dry weight (3.0 g cellulose) of Sigmacell α-cellulose per flask. The net mass of solids and solution in each flask was adjusted to 139 g with distilled water before adding citrate buffer (1.0 M, 7.5 mL) and sodium azide (10 g/L, 5 mL) solutions. Glacial acetic acid (1.1 mL) was then added to bring the pH to 4.8 before placing the samples in the incubator/shaker (50 °C, 100 rpm) to preheat for 15 min.

Each sample received 852 μL of cellobiase solution and 2040 μL of cellulase solution. The cellulase loading was equivalent to 62.5 FPU/g cellulose (25 FPU/g dry fiber). The samples were then returned to the incubator shaker in which the hydrolysis was conducted for 7 days at 50 °C. At the conclusion of the hydrolytic period, 20-mL aliquots were transferred to vials, heated in a boiling water bath for 15 min, and centrifuged. The supernatants were then analyzed by HPLC using the procedures described in Appendix C.

9.3. Results and Discussion

The average conversions for pretreated corn stover were (g anhydromonomer/g original polysaccharide): glucan, 0.880 g/g; xylan, 0.877 g/g; and arabinan, 0.921 g/g. The fact that the

the xylan had the lowest conversion suggests that the enzymes are incapable of hydrolyzing the linkages that occur between the hemicellulose backbone (xylan) and its non-sugar side-chains, such as 4-O-methylglucuronic acid, and/or lignin, thereby leaving some xylose encumbered. The average glucan conversion for α-cellulose was 98.9%.

10. Hydrolysis Time Profile

10.1. Introduction

The hydrolysis time profile indicates the progress of the enzymes and identifies the optimum hydrolysis time.

10.2. Materials and Methods

Two corn stover samples (7.5 g dry, -20+80 mesh) were pretreated according to standard procedure (0.125 g $Ca(OH)_2$/g dry biomass, 10 g H_2O/g dry biomass, 120 °C, 5 h) and transferred to 500-mL culture flasks such that the total mass of fiber and solution was 140 g; the lime loading was selected because it is on the plateau of the pretreatment curve (Figure 4) and removed from the shoulder occurring around 0.075 $Ca(OH)_2$/g dry biomass. The pH was adjusted to 4.8 with acetic acid before adding buffer, sodium azide solution, and enzymes as described for the standard enzymic procedure in Appendix A. The hydrolysis was conducted in the incubator-shaker (50 °C, 100 rpm) for 8 days. At time intervals of 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 days, 4-mL aliquots were removed, sealed in vials, heated in a boiling water bath for 10 min, centrifuged, and the supernatants analyzed by HPLC (Appendix C).

10.3. Results and Discussion

The conversions for glucan and xylan are plotted as a function of time in Figure 8. As can be seen in the figure, the optimum hydrolysis time at 50 °C using 5 FPU cellulase/g dry fiber is

about 60 h; the conversions under these experimental conditions were 0.545 g anhydromonomer/g polysaccharide and 0.519 g anhydromonomer/g polysaccharide for glucan and xylan, respectively. The conversions after 192 h for both glucan and xylan were about 0.6 g anhydromonomer/g polysaccharide.

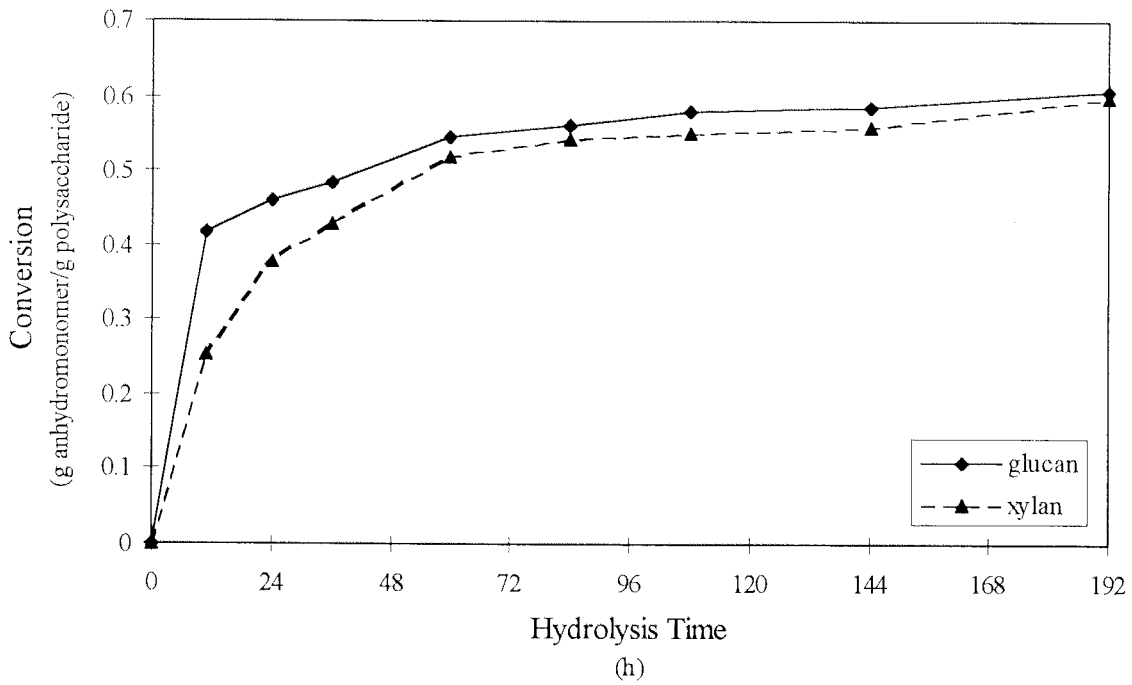

Figure 8. Hydrolysis time profile for enzymic saccharification of pretreated corn stover.

11. Simultaneous Saccharification/Fermentation

11.1. Introduction

The SSF experiments measure the effectiveness of the pretreatment for converting the corn stover glucan to ethanol.

11.2. Materials and Methods

NREL protocol No. 008 was followed except that 7.5 g of dry biomass (3 g cellulose) and 4.0 g dry Sigmacell α-cellulose (3 g cellulose) were hydrolyzed/fermented in a volume of 150 mL;

the volumes of inoculum (15 mL) and 10X YP (15 mL) were adjusted accordingly. The volume of cellulase added to each flask was 816 μL (25 FPU/g cellulose). The fermentation was performed at 38 °C using a shaker speed of 150 rpm. Duplicate samples of α-cellulose and pretreated corn stover were run concurrently. Intermittent samples were taken from the flasks at days 1, 2, 3, 4, 5, 6, and 7; these samples were centrifuged at 4000 rpm for 10 min after which the supernatants were withdrawn and kept frozen until analysis. HPLC analyses of the fermentation liquors were conducted according to the procedures described in Appendix C.

11.3. Results and Discussion

The results of the SSF are presented in graphical form in Figure 9. The ethanol yields averaged about 70% of theoretical for both pretreated corn stover and α-cellulose. The average pH of the fermentation liquors was 4.4 for corn stover samples and 3.9 for α-cellulose samples. Given that the glucose concentration remained at relatively low values throughout the SSF, the fermentation is clearly limited by the rate of cellulose hydrolysis, not by the metabolism of glucose by the yeast. Increasing the cellulase loading would undoubtedly improve the rate of fermentation.

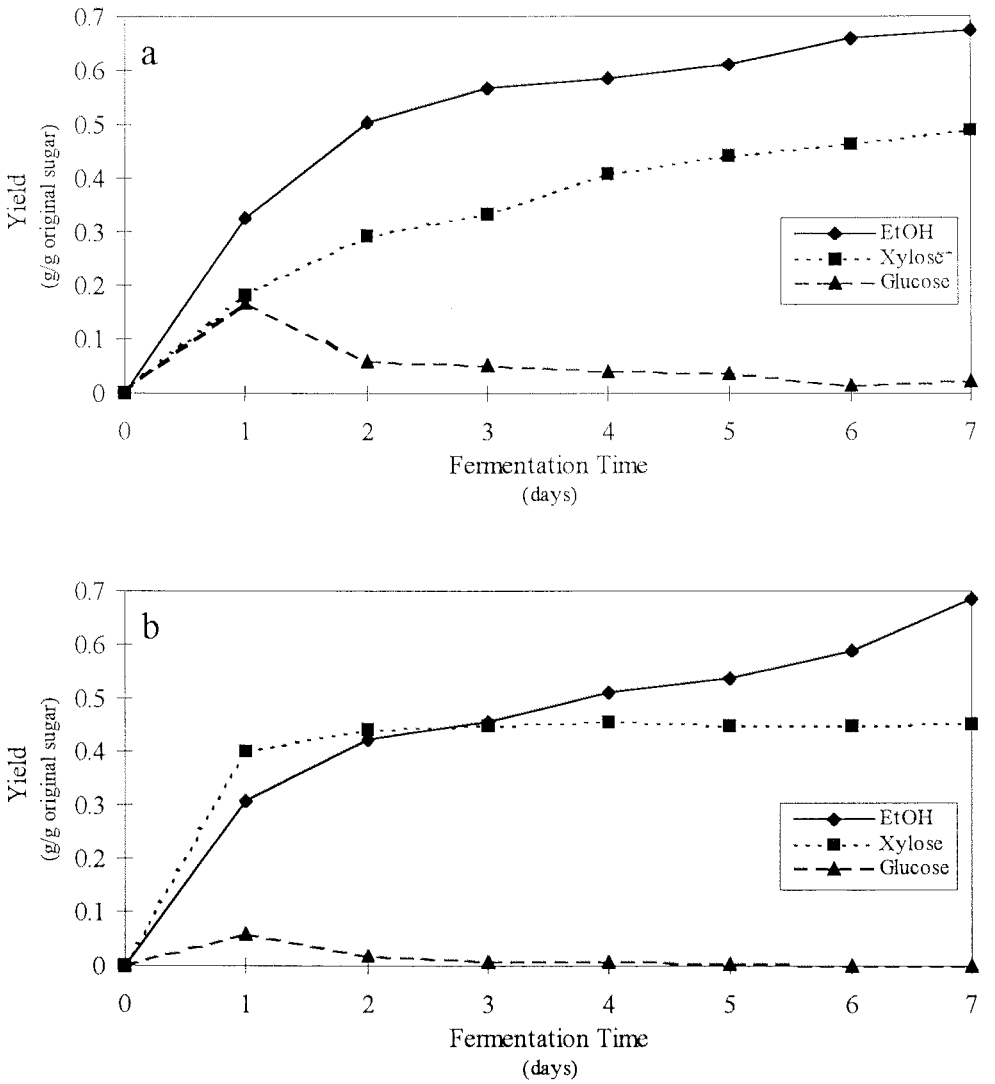

Figure 9. SSF results for pretreated corn stover (a) and *α*-cellulose (b) samples. Glucose and ethanol yields are g/g original glucose; xylose yield is g/g original xylose.

12. Summary and Conclusions

Lime pretreatment is effective for improving the enzymic hydrolysis of corn stover. For industrial purposes, the recommended pretreatment conditions are: lime loading 0.075 g $Ca(OH)_2$/g dry biomass; water loading 5 g H_2O/g dry biomass; and heating for 4 h at 120 °C. Washing the pretreated biomass before neutralization removed about 88% of the lime and reduced the acid required for neutralization by 50%.

The recommended enzyme loading for the enzymic saccharification of pretreated corn stover is 10 FPU/g dry biomass and the recommended hydrolysis temperature is 40 °C. The enzymic conversion of the corn stover to monosaccharides, when pretreated and saccharified as prescribed, was about 60% of the cellulose, 47% of the xylan, and 53% of the total available polysaccharide. Ultimate digestion of pretreated corn stover produced conversions of 88.0, 87.7, and 92.1% for the glucan, xylan and arabinan, respectively, compared to a glucan conversion of 98.9% for an α-cellulose control. Simultaneous saccharification/fermentation of α-cellulose and pretreated corn stover gave ethanol yields of about 70% of theoretical.

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APPENDIX A

STANDARD ENZYMIC PROCEDURE

1. Prepare 1-M citrate buffer by dissolving 201 g of citric acid monohydrate in about 800 mL distilled water in a 1000-mL volumetric flask. Adjust the pH to 4.8 using granular sodium hydroxide. Dilute the solution to the mark with distilled water and mix well by shaking.
2. Prepare 10 g/L sodium azide solution.
3. Place a 500-mL screw-cap flask on the pan balance. Insert a funnel in the top and tare the balance.
4. Remove one end of the reactor, mix the contents with a stirring rod, and pour the slurry into the funnel. Use additional water to effect the quantitative transfer of the sample into the flask. Bring the net mass of the sample to 140 g.
5. Add 7.5 mL of the citrate buffer using a 5-mL pipet; rinse the sides of the flask as the solution is added.
6. Add 5.0 mL of the sodium azide solution. Again, rinse the sides of the flask with the solution.
7. Add glacial acetic acid to the flask, with a buret, to pH 4.8.
8. Attach the cap and place the flask in the incubator-shaker (Amerex Instruments Orbital Shaker Incubator #GM706; Hercules, CA). Adjust the shaking speed to 100 rpm. Allow at least 10 min for the solution to reach equilibrium.
9. Add the Tween and/or water, then 0.852 mL ß-glucosidase (Novozyme 188 cellobiase, 213 IU). Add 0.408 mL (5 FPU/g, 7.5-g basis) cellulase (Spezyme-CP) last.
10. Return the sample to the incubator-shaker.

APPENDIX B

DNS ASSAY PROCEDURE

DNS Reagent Preparation

1. Dissolve 10.6 g of 3,5-dinitrosalicylic acid (DNS) crystals and 19.8 g sodium hydroxide in 1416 mL of distilled water.
2. Add 306 g Na-K-tartrate (Rochelle salts).
3. Melt phenol crystals under a fume hood using a water bath at 50 °C. Add 7.6 mL of phenol to the above mixture.
4. Add 8.3 g sodium meta-bisulfite ($Na_2S_2O_4$).
5. Add NaOH, if required, to adjust the solution pH to 12.6.

DNS Sample Preparation

6. Pipet 0.5 mL of clear hydrolyzate into a 25-mL volumetric flask.
7. Add distilled water to the mark, and mix well by shaking.
8. Using some of the diluted sample, rinse a 20-mL scintillation vial. Add about 5 mL of solution to the rinsed vial.
9. Pipet 0.5 mL of the diluted sample into a 22-mm screw cap test tube.
10. Pipet 1.5 mL of DNS reagent (above) into the test tube.
11. Attach the cap and mix the sample by vortexing.
12. Repeat Steps 4-6 for distilled water (blank) and glucose standards.
13. Place the samples in a boiling water bath for 15 min.
14. Allow the samples to cool before adding 8 mL of distilled water.

15. Vortex the samples and measure the absorbance at 550 nm (Milton Roy, Spectronic 1001). Use the water blank sample to zero the absorbance of the spectrophotometer, and the glucose standards to prepare a calibration curve.

APPENDIX C

HPLC ANALYSIS PROCEDURE

General

1. Prepare 0.01-M sulfuric acid mobile phase by adding 2.72 g of 72% H_2SO_4 to 2 L of distilled water. Filter the solution by vacuum through a 0.2-μm nylon filter and degas the solution by boiling under vacuum.
2. Keep the mobile phase on a hot plate to maintain a temperature of 85 °C to prevent the adsorption of air. Prime the pump (LDC Constametric 3200; Riviera Beach, FL) with the fresh mobile phase.
3. After assuring that all air bubbles have been removed from the system, adjust the pump flow rate to 0.2 mL/min and connect the column (Bio-Rad HPX-87H; Richmond, CA).
4. Close the lid of the column heater over the column, turn it on, and set the temperature to 50 °C.
5. Once the temperature has reached 50 °C, slowly (0.1 mL/min/min) ramp up the flow rate to 0.6 mL/min.
6. Adjust the energy of the refractive index (RI) detector (LDC Refractomonitor III) to about 5.
7. Monitor the baseline of the integrator (Spectra Physics SP4270; Fremont, CA). Once the baseline stabilizes, zero the detector and begin analyses.

Clean-up Column

1. Prepare CO_3^{-2}-form anion exchange resin. This is best accomplished by filling a suitable liquid chromatography column (preferably about 500 mL in size) with Bio-Rad AG IX-8 100-200 mesh Cl^- resin. Pass about 20 column volumes of 1-M NaOH solution through the resin bed, followed by about five column volumes of water; do not allow the flow rate to go higher than 3 mL/cm^2-min. Now pass 20 column volumes of 1-M Na_2CO_3 solution through the resin bed, followed by 10 column volumes of water. This completes the preparation of the anionic exchange resin.
2. Rinse some Bio-Rad AG 50W-X8 100-200 mesh H^+ form cation exchange resin with distilled water.
3. Place the tip on a Bio-Rad 10-mL Econocolumn and fill to the shoulder with distilled water.
4. Add cation exchange resin to the 0.2-mL line on the column graduations. Allow the resin to settle to a flat bed.
5. Add anion exchange resin to the 0.6-mL line (0.4 mL of resin).
6. Allow the water to drain out, and rinse the resin bed with about 1 mL of distilled water. This completes the preparation of a clean-up column.

Enzymic Hydrolyzates

1. Pipet 0.5 mL of clear hydrolyzate and 0.5 mL of a 10-mg/mL erythritol internal standard solution into a 4-dram vial. Attach the cap and mix the solution by shaking.
2. Position a labeled autosampler (Spectra Physics AS100) vial under the tip of a clean-up column.
3. Apply the mixture to the resin bed using a transfer pipet.

4. Collect the eluant. Pipet about 0.5 mL of distilled water onto the resin bed as a rinse; collect this into the vial as well. Put the cap on the vial and shake vigorously. The sample is ready for injection into the HPLC system.

Acid Hydrolyzates

1. Pipet 25 mL of hydrolyzate into a 100-mL beaker. Add 1 mL of 10-mg/mL erythritol internal standard solution, and adjust the pH to about 4 with saturated $Ba(OH)_2$ solution.
2. Decant the solution into a 50-mL centrifuge tube, and centrifuge for 5 min at 3500 rpm.
3. Pour the clear supernatant into a 100-mL round bottom flask, and concentrate the sample to about 2 mL using a rotary evaporator (Büchi). During the concentration step, heat the sample to 40 °C in a water bath.
4. Apply the concentrate to a clean-up column, as described for the enzymic hydrolyzates. Collect the eluant and rinse in a 4-dram vial.
5. Mix the prepared sample well before transferring a suitable amount to an autosampler vial.

APPENDIX D

ACID HYDROLYSIS PROCEDURE

Acid Preparation

1. Fill a large (at least 2-gallon) bucket with ice water.
2. Pour about 150 mL of concentrated sulfuric acid into a 250-mL graduated cylinder. Place the cylinder into the bucket with the ice water.
3. Carefully dilute the acid with cold water. Monitor the specific gravity of the acid with a hydrometer. Allow ample time for the acid to cool between additions of water. Periodically, decant the acid into a beaker to ensure complete mixing.
4. Dilute the acid to a specific gravity of about 1.635 at 15°C. Transfer to an airtight container with a Teflon-sealed cap.

Hydrolysis

1. Dry some -40 mesh material in the oven at 105 °C; don't allow the sample to remain in the oven longer than overnight.
2. Weigh 100 mg of the dry biomass to 0.1 mg accuracy. Quantitatively transfer the dry biomass into a heavy-duty, 50-mL, glass screw cap centrifuge tube (Korex). Try not to allow any biomass to adhere to the sides of the tube. It is helpful to have the centrifuge tube in a rack during the course of the acid digestion.
3. Pipet 893 μL of 72% sulfuric acid onto the dry biomass. Use a six-inch glass stirring rod to mix the slurry and disintegrate the larger particles.

4. Place the tube containing the sample into a water bath at 30 °C. Periodically stir the sample over the course of 1 h.
5. After 1 h, pipet 25 mL of distilled water into the tube. Be sure to completely rinse the stirring rod as the water is added.
6. Attach the cap firmly to the tube, and place the sample into an autoclave. Steam the sample at 120 °C for 45 min. Be sure not to vent the autoclave at the conclusion of the steaming time; allow the autoclave to cool to atmospheric pressure before opening the autoclave door.
7. Filter the sample through a tared, 15-mL sintered glass filter (medium). Use about 10 mL of water to rinse the solids retained on the filter. Place the filter in the oven to dry. Quantitatively transfer the filtrate to a 50-mL volumetric flask. Use water sparingly to rinse the vacuum flask and centrifuge tube. Dilute to the mark with distilled water. The sample is now ready for processing according to the procedure described for acid hydrolysed samples in Appendix C.
8. Pipet 1 mL into a 25-mL volumetric flask and dilute to the mark with distilled water. Measure the absorbance at 205 nm against a sulfuric acid blank (appropriate concentration). Calculate the acid soluble lignin assuming an absorptivity of 110 L/g-cm.
9. Obtain a large quantity (about 2 g) of Klason lignin by repeating Steps 1-7 using about 1.5 g dry biomass and 10 mL 72% sulfuric acid per 600-mL beaker; about six beakers (9 g dry biomass) should be adequate. The dilution step requires 280 mL of distilled water per sample. Cover the beakers with Petri dishes during the autoclaving step. Once the Klason lignin is dry, use it to determine the ash content correction for the lignin measured in Step 7.